IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application No.	:	10/806,253
First Named Inventor	:	David C. BAULCOMBE
Filed	:	March 22, 2004
TC/A.U.	:	1638
Examiner	:	N/A
Docket No.	:	101044.53943D1
Title	:	Gene Silencing

STATEMENT REGARDING INFRINGEMENT OF PENDING CLAIMS IN SUPPORT OF PETITION TO MAKE SPECIAL

Mail Stop =====
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313 1450

Sir:

I, Gerard Bencen, declare and state as follows:

1. That I am currently employed by Plant Biosciences Limited (PBL) a life sciences company located in the United Kingdom as Patent Manager.

2. That PBL was established in 1994 by the Gatsby Charitable Foundation and the John Innes Center. PBL is a for-profit technology company specializing in life sciences, including plant and microbial technology.

3. That PBL's operational activities center on protecting the intellectual property assets of PBL's two founder institutes and more recently to manage and protect the IP assets of other research laboratories and Universities located predominantly but not exclusively in the United Kingdom.

4. That in 2004 the Biotechnology and Biological Sciences Research Council (BBRSC) became a shareholder in PBL further establishing PBL's status in the UK as a company whose mission is exploiting new biotechnologies arising from public funding and emerging life science technologies.

5. That the technology being pursued in this application and related patent applications currently on file at the USPTO, which are directed to detection of small RNA molecules (SRMs) as a marker that post-transcriptional gene silencing (PTGS) is occurring in the cells of an organism is a major asset of the company.

6. That, in my opinion, the development of this technology will be significantly impaired if examination of this application is delayed. A delay of the issuance of this application will significantly impact PBL's ability to commercialize and license its technology in part because of the fact that PBL is aware of a large number of entities who are infringing the invention claimed in this application. Such entities include Ambion, which sells a kit specifically to detect the presence of SRMs in the cells of an organism (Exhibit A). We are also aware that Kreatech has launched its first miRNA/siRNA isolation and labeling kit. See the fourth full paragraph of the Press release attached hereto (Exhibit B). Additionally, researchers and pharmaceutical companies developing PTGS based therapies are infringing the methods claimed in this application (see, for example, Anderson and Akkina, 2005 (Exhibit C) attached hereto, where the production of short interfering RNA molecules is confirmed in Figure 5 in Magi-X4 cells and Ghost-R5 cells, in experiments aimed at defining a PTGS-based anti-HIV treatment). Also see Exhibit D,

a research paper entitled "A Simple and Rapid method to detect plant siRNAs using Nonradioactive Probes by Goto et al. which appeared in Plant Molecular Biology Report 21:51-58 (2003). The method disclosed entails isolating nucleic acid from plants where silencing is occurring and characterizing the sequences of the isolated short RNA molecules using sequence specific probes. Exhibit E, a manuscript by Kawasaki et al. describes methods for detection of siRNA which fall within the scope of the present claims. PBL believes that the use of the Ambion kits and detection of SRMs as evidenced in the work of Anderson and Akkina 2005, the Kreatech methods and kits and the methods disclosed by Goto and Kawasaki for example, give rise to infringement of the claims being pursued in this application.

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that the statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

27 MARCH 2006
Date

Gerard Bencen
Patent Manager
Plant Biosciences Limited

TechNotes 10(4)

Detecting Attomole Amounts of Small RNA *mir*Vana™ miRNA Detection Kit

•Sensitive -- Detect miRNA or siRNA in as little as 10 ng total RNA

•Specific -- Extremely low background with no need to optimize hybridization or washing conditions

•Simple and fast -- Single tube procedure eliminates the need to transfer to membrane for hybridization

•Multiple target detection-- Detect small RNAs and mRNAs in the same sample

The new *mir*Vana miRNA Detection Kit provides a faster and more sensitive alternative to Northern analysis for detecting small RNAs. With this patented technology, quantitation of small RNA species can be performed with as little as 10-50 ng of total RNA. The assay is incredibly sensitive and is able to detect attomole (10^{-18} mol) amounts of target RNA. In addition, the *mir*Vana miRNA Detection Kit can be used to simultaneously detect several small RNAs of the same size or both small RNA and long RNA species in the same sample. This means you can now quantitate an siRNA and its target mRNA in the same sample. (See several applications of this kit in ***Detecting miRNA & siRNA***.)

Rapid Procedure Based on Solution Hybridization

The *mir*Vana miRNA Detection Kit is based on a simple solution hybridization principle. The sample RNA containing the target RNA(s) of interest is mixed with one or more high specific activity radiolabeled RNA probes and the included Hybridization Buffer. After heat denaturation, each mixture is incubated at 42°C to hybridize the probe to its complementary RNA molecules. Unhybridized RNA species and excess RNA probe is then removed by a rapid ribonuclease digestion step. The hybridized, protected RNA fragments are recovered in the same tube using Ambion's patented single step technology for simultaneous ribonuclease inactivation and nucleic acid precipitation. RNA samples are then resuspended with the provided Gel Loading Buffer and analyzed on a denaturing polyacrylamide gel. Because the hybridization is performed in solution, the procedure ensures a sensitive and linear detection signal after autoradiography. Furthermore, the included Hybridization Buffer has been specifically developed to provide the optimal sensitivity and specificity of detection with short antisense probes. Thus the procedure is ideal for detecting small RNA molecules such as siRNA or miRNA.

Complete Kit with Included Controls

Each *mir*Vana miRNA Detection Kit provides enough reagents for 100 reactions. The kit also contains a control transcription template and Probe Elution Buffer to prepare and gel purify a 32 nt RNA probe specific for miR-16 miRNA. When used with the provided Mouse Kidney Total RNA, this probe generates a 22 nt protected fragment. The kit also comes with a detailed Instruction

TechNotes

Ordering Information

Related Links:
Detecting miRNA & siRNA
[read]

Prepare siRNA and miRNA Probes in Just 1 Hour
[read]

New Tools for miRNA and siRNA Analysis
[read]

miRNA Resource
[read]

RNA Isolation Can Affect miRNA Analysis

Recent studies suggest that microRNAs (miRNAs) may play an important regulatory role in gene expression, especially during differentiation and development. Many miRNAs are expressed at differing levels across tissues and developmental stages. When studying miRNAs in tissues or cells, choosing a compatible RNA isolation method is critical. Not all isolation procedures provide quantitative recovery of small RNAs. In fact, one of the most common methods utilization of a silica matrix or glass fiber filter can result in the loss of almost all small RNAs. Figure 1 (below left) shows a comparison of three traditional methods of RNA isolation: a single-phase phenol based method (RNAwiz™), a glass fiber filter method (RNAqueous®), and a guanidinium/double phenol extraction method (ToTALLY RNA™). The data demonstrate that miRNAs are recovered by both phenol-based methods while the standard glass fiber filter method yields virtually no detectable miRNA. This suggests that, while perfectly suitable for isolating messenger RNA, traditional glass fiber filter based methods are not appropriate for the

Manual and comprehensive information about experimental setup and probe design/preparation.

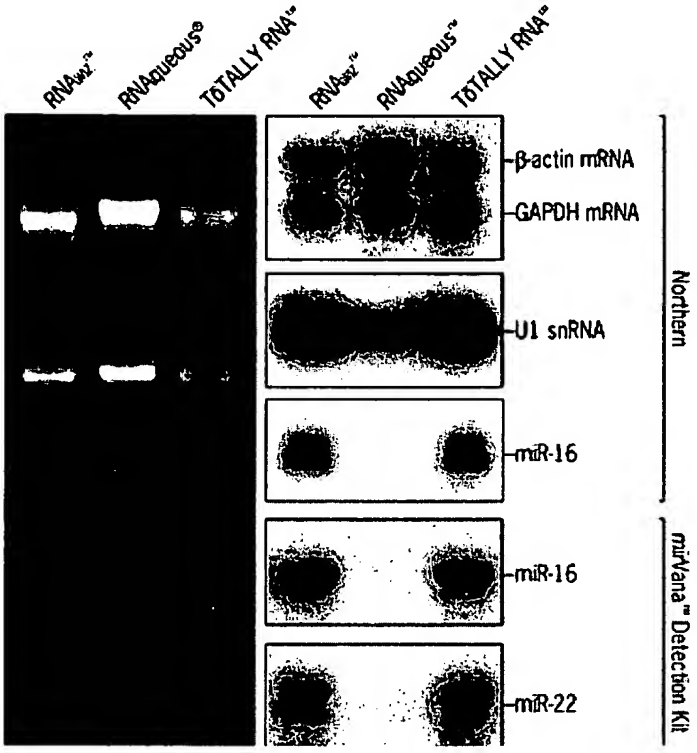

Figure 1. Differential Recovery of Small RNAs During Total RNA Isolation. Total RNA was isolated from 1x10^6 HeLa cells using three standard RNA isolation procedures: single phenol/guanidinium extraction (RNAwiz™), glass fiber filter (RNAqueous®) purification, or double phenol extraction (ToTALLY RNA™). Purified RNA samples were analyzed on a 1% denaturing agarose gel and recovery of messenger RNA (β-actin, GAPDH), small nuclear RNA (U1 snRNA), and miRNA (miR-16, miR-22) were assessed by Northern blot or solution hybridization with the *mir*Vana miRNA Detection Kit.

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Ordering Information

Cat#	Product Name	Size
1552	*mir*Vana™ miRNA Detection Kit	100 rxns

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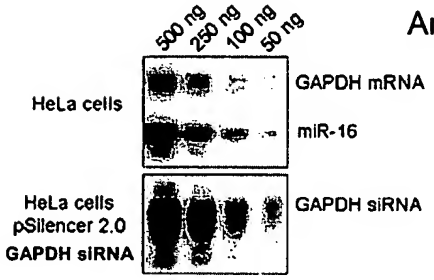
recovery of small RNAs including miRNAs.

Total RNA Does Not Always Contain miRNA

When purchasing commercially available purified RNAs it is also important to consider the isolation method used to produce the RNA. If the RNA was prepared using traditional glass fiber filter methodologies, it most likely does not contain detectable levels of miRNAs. Ambion has developed optimized protocols for isolating RNA from various tissues, and we are currently evaluating each of our FirstChoice® Total RNA products for the presence of miRNAs. This includes RNA from over 80 different human, mouse, and rat tissues. Please click here for a list of "miRNA Certified" FirstChoice RNAs.

Isolation & Detection of Small RNA Molecules (siRNAs, miRNAs)



Ambion, Inc. ~ The RNA Company
techserv@ambion.com
www.ambion.com

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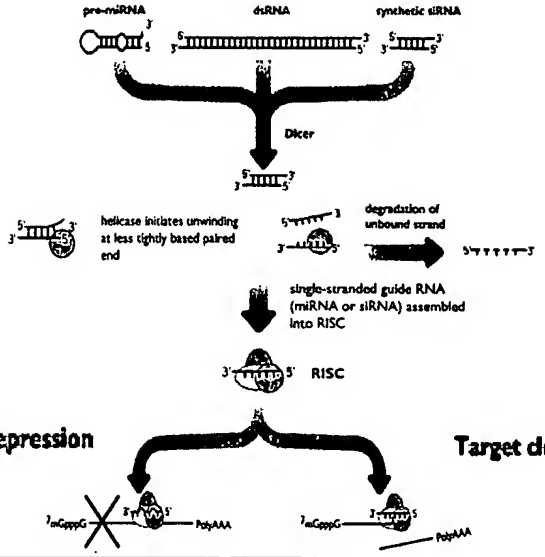
Gene Regulation: miRNAs & siRNAs



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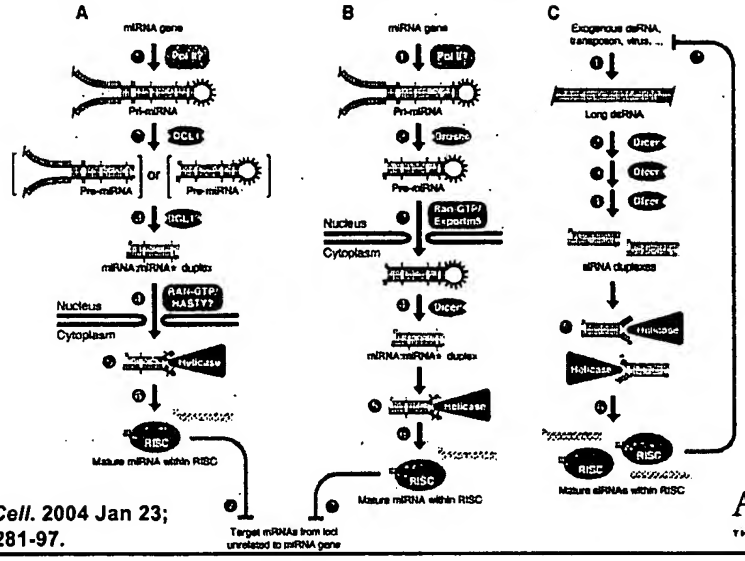
The microRNA Pathway



Bartel. *Cell.* 2004 Jan 23; 116(2): 281-97.

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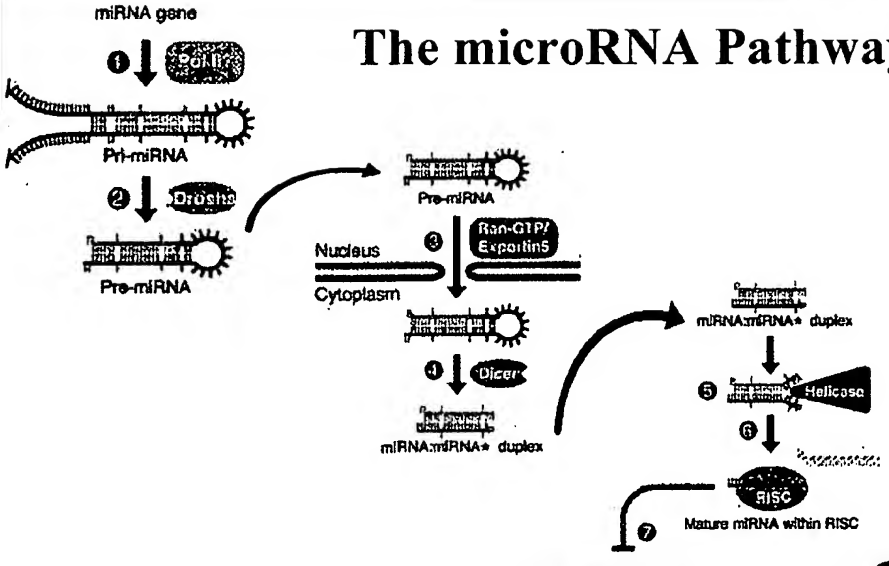
The microRNA Pathway



Bartel. *Cell.* 2004 Jan 23; 116(2): 281-97.

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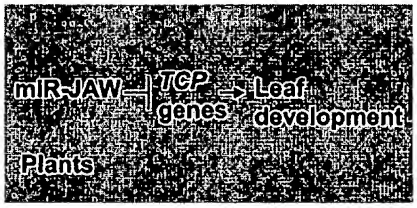
Examples of miRNA Functions

Ambros (2003). *Cell* 113:673-676.

Brennecke et al (2003). *Cell* 113:25-36.



Palatnik et al (2003). *Nature* 425:257-263.

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Additional Functions of Small RNAs

- Antiviral defense
- Transposon silencing
- Gene regulation via chromatin condensation
- Genomic rearrangements

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siRNA Applications

- Transient downregulation (siRNA)
- Long-term knockouts (vectors)
- Functional genomics
- Pathway elucidation
- Drug target identification and validation

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Small RNA Analysis

Analysis of:

Native small RNA molecules
miRNAs, siRNAs, snRNAs, snoRNAs, etc.

Exogenous small RNAs
siRNAs, hairpin siRNAs, or miRNAs that are introduced or expressed in cells or in animals (e.g. tail injection, expression in cells or transgenic animals, etc.)

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Challenges to Small RNA Analysis

The detection procedure:

- RT-PCR
  - -Cannot use standard RT-PCR
- Northern blots
  - -Insensitive (10-50 μg RNA, long exposure)
  - -Single target detection
  - -Time consuming

The RNA isolation method:

- Loss of small RNAs

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Detection of small RNAs

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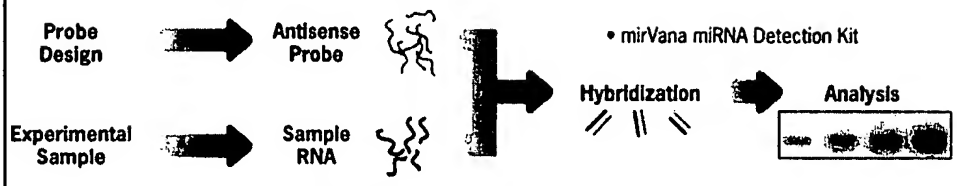
Analysis of Small RNAs

- mirVana Probe & Marker Kit
- mirVana miRNA Probe Construction Kit



- Validated FirstChoice Total RNAs
- mirVana miRNA Isolation Kit

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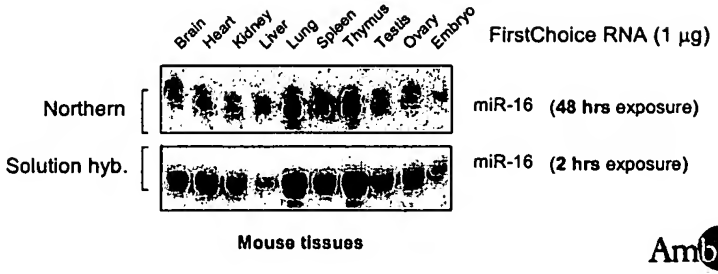
New Assay for Detecting Small RNAs

Features of the assay:

- Sensitivity
- Specificity
- Versatility



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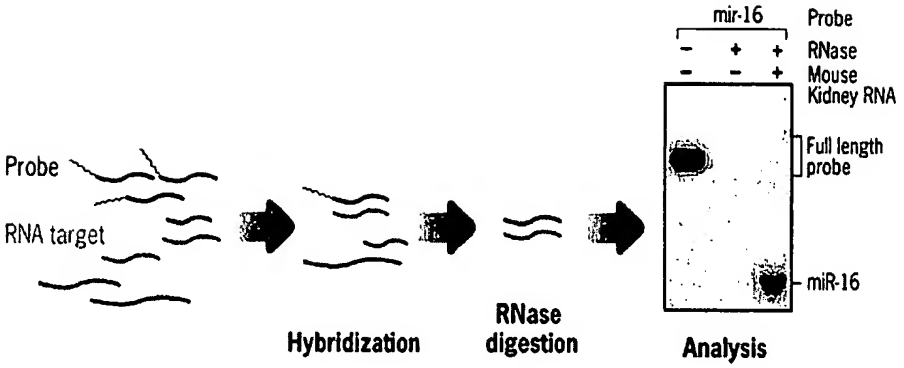
The Solution Hybridization Assay



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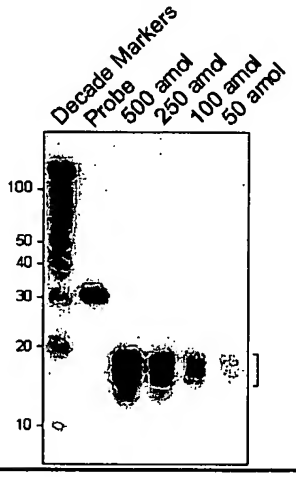
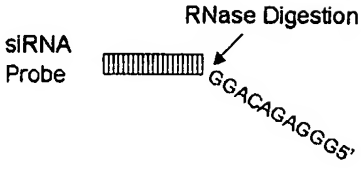
Sensitivity of Detection

Can detect ~50-100 amol (2 hrs. exposure)





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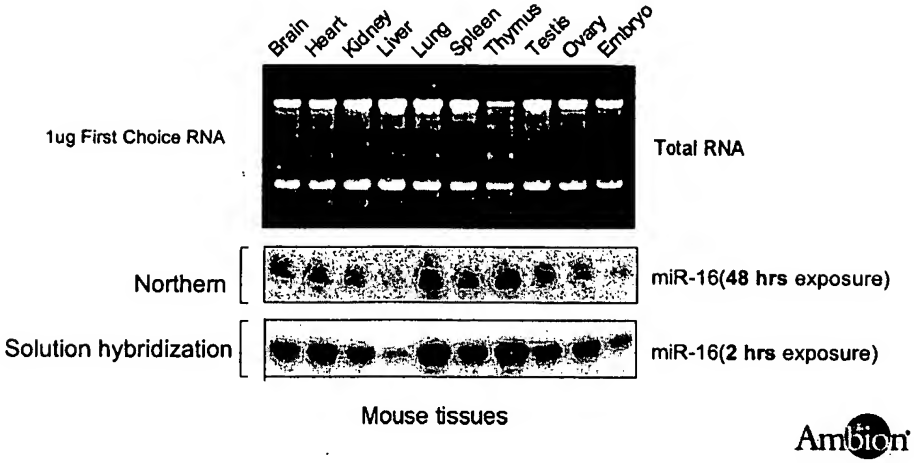
Comparing Detection Methods



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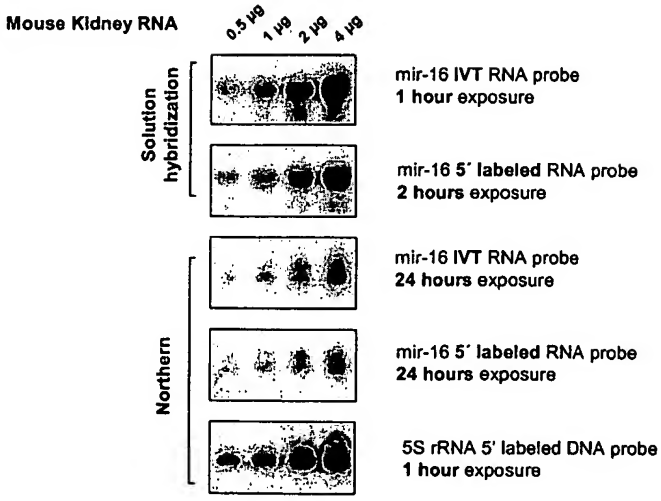
Sensitivity vs. Northern Blot



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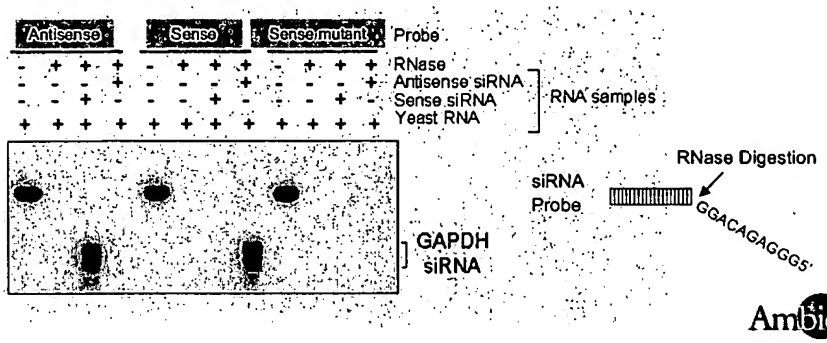
Specificity of Detection

- Spiked 200 amol GAPDH siRNA (either sense or antisense strand) into 4 ug yeast RNA
- Detected each siRNA strand with the corresponding probe



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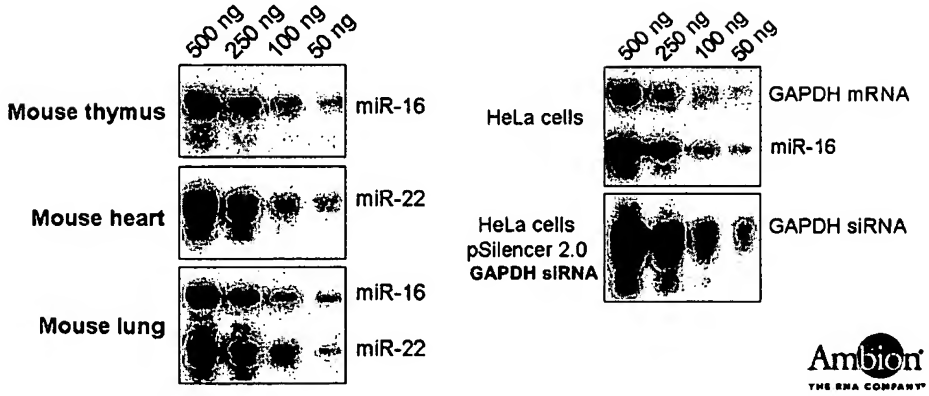
Versatility of the Assay

- Total RNA from mouse tissues
- 3.5 hrs exposure



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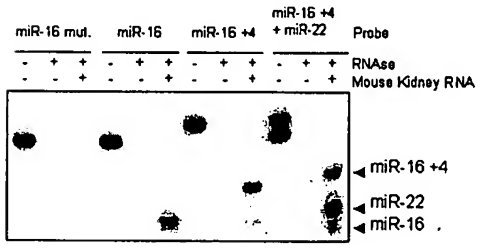
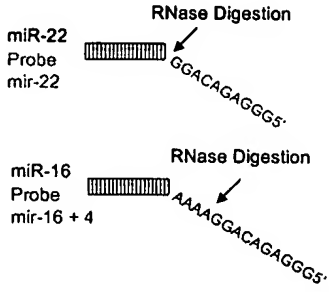
Versatility: Multiple miRNAs

- Northern blots cannot simultaneously detect multiple miRNAs of the same size
- Solution hybridization assay can





Add several A residues to the miR-16 probe to detect miR-16 and miR-22 simultaneously by solution hybridization assay

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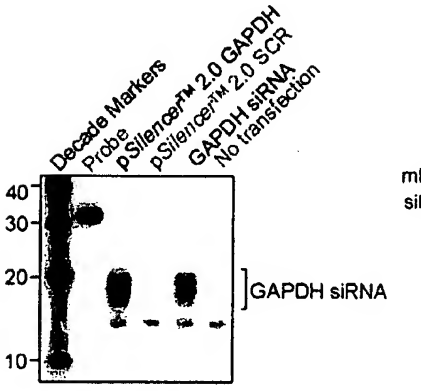
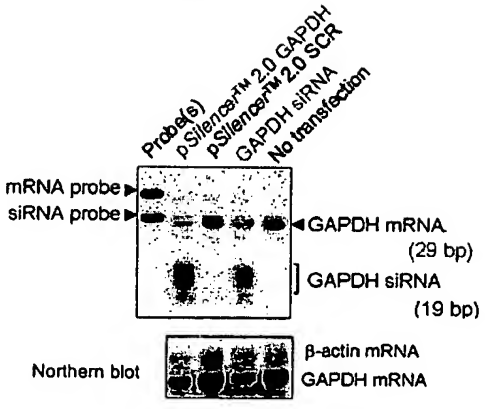
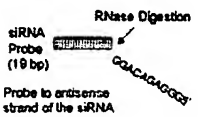
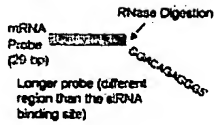
Versatility: Multiple Targets









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Importance of Detection Method

Expression across species using Northern blots

	miR-16	miR-20	miR-26
HeLa	+++	+++	+
mouse kidney	+++	-	+++
Fish	+	+	+++
Frog ovary	+	-	-
S2	-	-	-

Lagos-Quintana et al (2001). *Science* 294:853-858.

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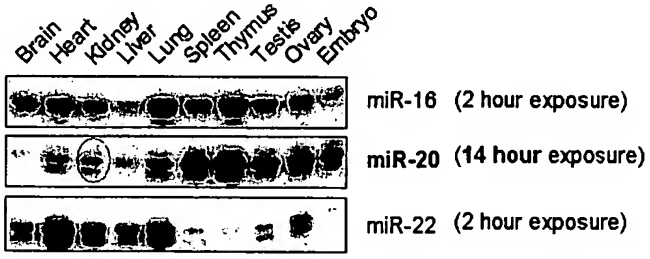
Importance of Detection Method

- Detection of miRNAs from 1μg of total RNA
- Unlike previous findings, miR-20 was detected in mouse kidney total RNA



Mouse tissues

Solution hybridization assay

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RNA Isolation: Capturing small RNAs

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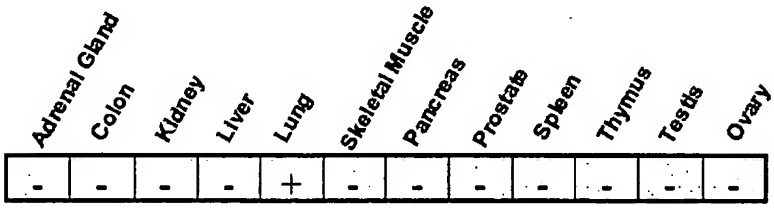
Importance of RNA Isolation

Expression of miR-200b across human tissues using Northern blots



Grad et al (2003). *Mol Cell* **11**:1253-1263.

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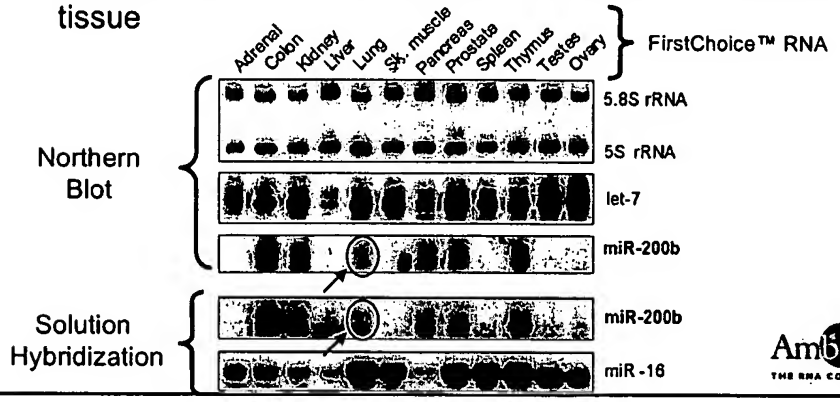
Importance of RNA Isolation

- Unlike previous findings, miR-200b was detected in colon, kidney, pancreas, prostate, and thymus
- May be due to RNA source and developmental stage of tissue



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RNA Isolation Methods

1. Phenol/chaotropic reagent extraction

Disrupt tissue in chaotropic reagent/acid phenol/chloroform → Phenol extract → Centrifuge and remove supernatant → precipitate RNA

2. Glass fiber filter

Disrupt tissue in chaotropic reagent → Bind RNA to glass fiber filter → Wash unbound DNA, protein, salt, nucleotides, etc. → elute the RNA

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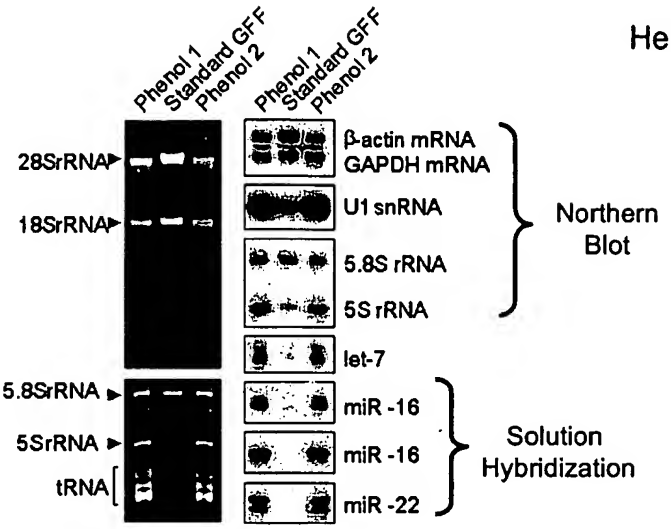
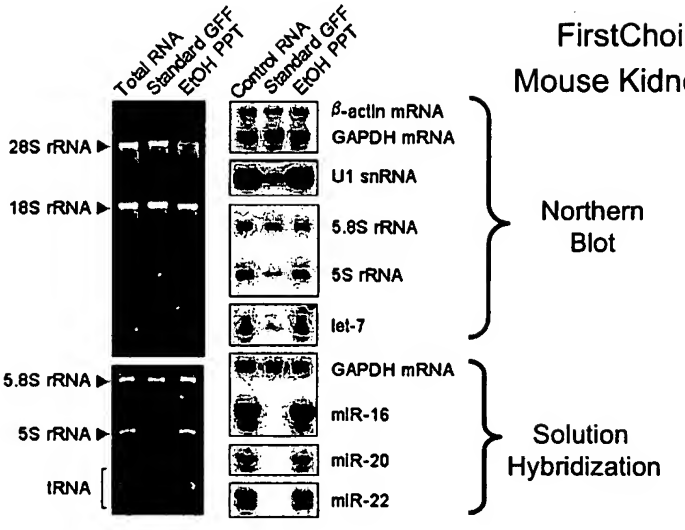
Phenol-based vs. Standard GFF

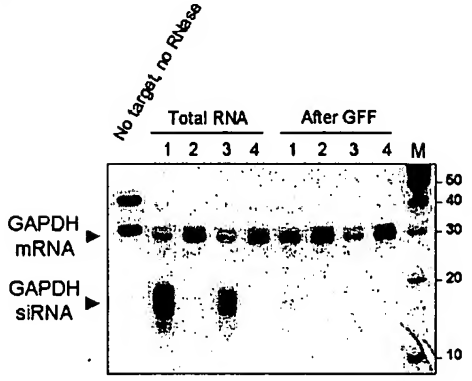


Loss of Small RNAs

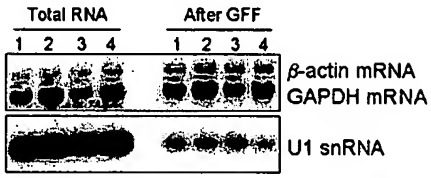


Loss of siRNAs

RNA isolation method is important for recovery of siRNAs



LANE	SAMPLE
0	No target, no RNase
1	pSilencer 2.0 GAPDH
2	pSilencer 2.0 SCR
3	GAPDH siRNA
4	No transfection



Solution Hybridization

Northern Blot

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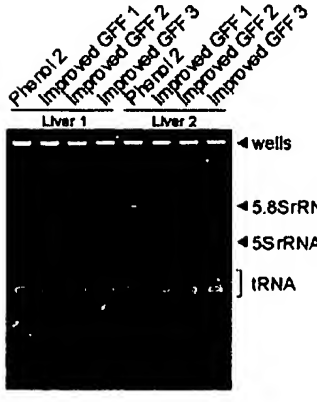
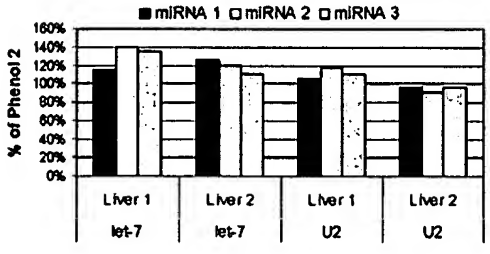
Improved GFF Protocol

- EtBr staining
- 1µg mouse liver total RNA
- Northern blot analysis
- Phosphorimager quantitation





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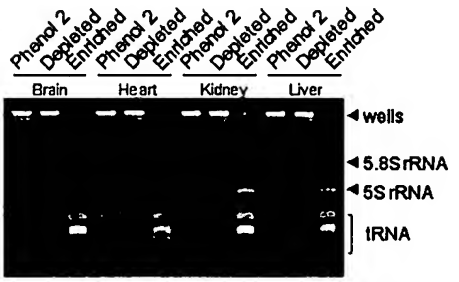
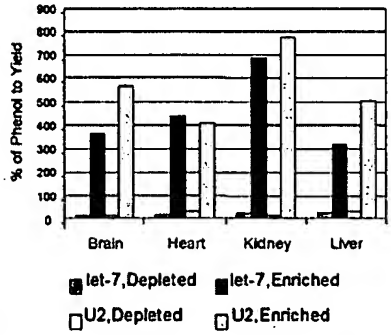
Enrichment of Small RNAs



- Northern blot analysis
- Phosphorimager quantitation



- Enrichment of small RNAs (<200 nt)
- Carried out using improved GFF protocol
- 1 μg RNA in each lane

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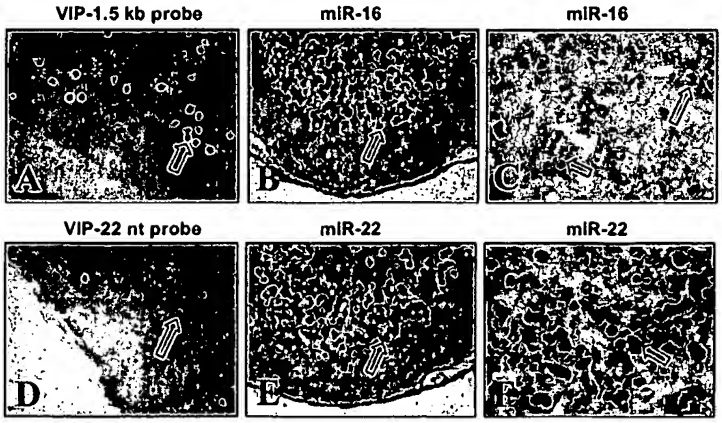
In Situ Hybridization of miRNAs



Mouse brain cortex (A,B,D,E)
Head of the caudate nucleus (C,F)

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Summary

Detection:

- Solution hybridization
  - Sensitive
  - Multi-target detection (siRNA and mRNA)
- Northern blots
- In situ hybridization

RNA isolation:

- Phenol-based methods
- Modified GFF (enrichment)

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Tools for Small RNA Analysis

*mir*Vana™ miRNA Detection Kit

*mir*Vana™ miRNA Probe Construction Kit

*mir*Vana™ miRNA Isolation Kit

*mir*Vana™ miRNA Probe Labeling & Marker Kit

mRNA*locator*™ In Situ Hybridization Kit

FirstChoice® Total RNA (human, mouse, rat tissues)

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*mir*Vana™ miRNA Detection Kit

- Reagents for 100 solution hybridization assays
- Control dsDNA template for generating mir-16 probe
- Enough for generating 10 probes by in vitro transcription
- Control Mouse Kidney Total RNA (10 μg)

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*mir*Vana™ miRNA Probe Construction Kit

- Reagents for 30 transcription reactions, including reagents for:
  - template preparation (T7 promoter primer, dNTPs, Klenow, etc.)
  - in vitro transcription (T7 enzyme, NTPs, etc.)
- Control DNA primer for generating mir-16 + 4 probe

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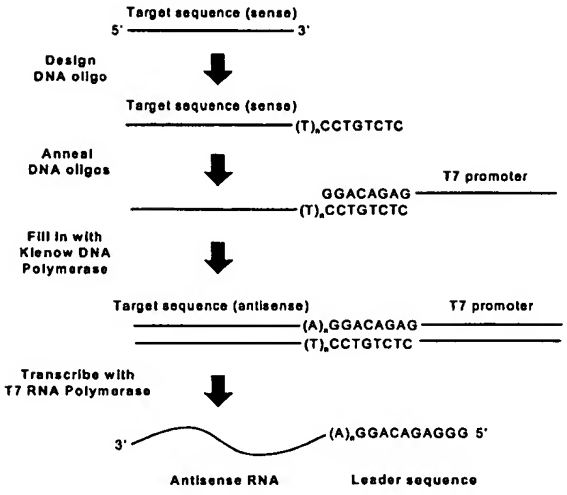
*mir*Vana™ miRNA Probe Construction Kit



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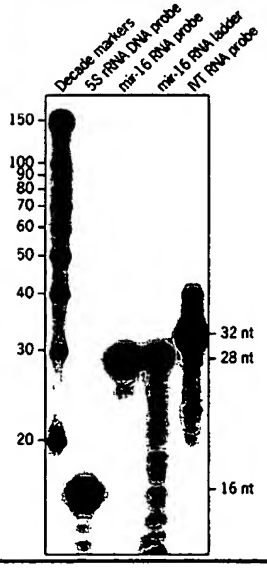
*mir*Vana™ miRNA Probe & Marker Kit



- T4 PNK (30 rxns) + RNA and DNA purification
- Decade™ Marker (10 rxns)
  150, 100, 90, 80, 70, 60, 50, 40, 30, 20, 10 bp
- Alkaline Hydrolysis Buffer to generate a ribonucleotide single base ladder
- Control probes
  -Control RNA Probe (miR-16)
  -Control DNA Probe (5S rRNA)

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*mir*Vana™ miRNA Isolation Kit

Reagents for 40 Total RNA isolations or 20 enrichment/depletion experiments

- 40 glass fiber filters
- Lysis solution
- Wash solutions
- Elution solution

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FirstChoice® Total RNA

- RNA from over 80 different human, mouse, and rat tissues
- For a list of "miRNA certified" FirstChoice RNAs, please visit http://www.ambion.com/prod/fcrna

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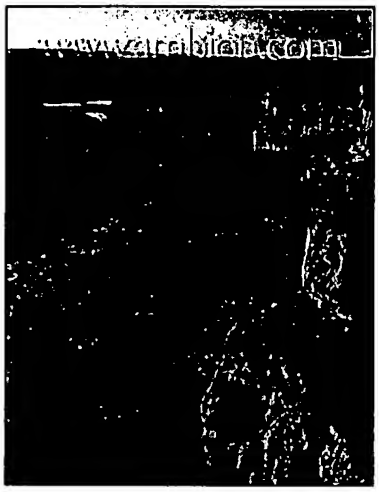

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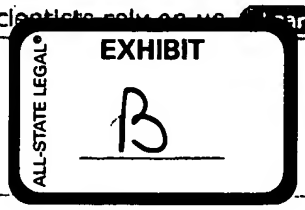

Kreatech launches first miRNA isolation labelling ki

By Wai Lang Chu

14/03/2006 - **Kreatech has launched the first complete miRNA isolation and labelling kit, which combines the upstream isolation of small RNA and the reagents necessary to label these DNA samples for microarray analysis, offering a more complete miRNA analysis solution.**

The discovery of small RNAs that have regulatory effects on gene expression has excited researchers to further characterise the function of these molecules in molecular processes and as possible tools in drug discovery.

One common way to examine these small RNAs, either siRNAs or miRNAs, is to analyse them on a microarray. Due to their small size, these RNA molecules prove very difficult to label via the enzymatic reactions common to NHS-ester chemistry.

Kreatech's miRacULS miRNA isolation and labelling kit provides a solution to isolate and label miRNAs as well as siRNAs. A 30-minute column-based isolation is followed by a one step, 15 minute; direct labelling without the need for enzymes.

This kit also includes the proprietary KREApure purification columns. These columns were developed to specifically remove unreacted ULS reagent from the labelling reaction.

The KREApure columns have no affinity for nucleic acids and for this reason the recovery is extremely high as fragments of all sizes flow through, making it ideal for work with miRNA samples.

"Our miRacULS miRNA isolation and labelling kit expands our product offering for microarray based miRNA and siRNA analysis. By supplying to the market the first combined isolation and labelling kit we provide our customers with convenient, rapid and reproducible kits to carry out their miRNA experiments," said Brent Keller, General Manager of Kreatech USA and VP Commercial Operations.

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The market for gene expression analysis is dominated by three distinct technologies: microarray analysis, quantitati (qPCR) and RNA interference (RNAi).

Though estimates vary greatly, the market for tools that aid in studying genetic expression figures in the billions eve

According to Frost and Sullivan, pre-made commercial microarrays, supplies for self-printed microarrays, and microa software brought in revenues worth $596 million in 2003. That figure is estimated to grow to $937 million by 2010, annual growth rate of 6.7 per cent.

Efforts to understand the genome have recently received a big boost from the refinement of RNA inhibition, in which double-stranded DNA introduced into a cell undergoes a natural process to silence expression of the mRNA with a co sequence.

Worldwide, the RNAi market brought in revenues of $48 million in 2003. With an annual growth rate of 31.5 per cen expects to bring in $358 million by 2010.

At that point, use of RNAi for target validation is estimated to generate $146.4 million with its use in basic research million. Over the long term, the purchase of RNA oligonucleotides is estimated to generate 56 per cent of the marke based siRNA may be responsible for 18 per cent.

The miRacULS miRNA Isolation and Labelling kit is available with the ULS labelling molecule bound to Cy3 and Cy5 d GE Healthcare.

Since the ULS labelling technology is a non-enzymatic labelling reaction whose labelling efficiency is independent of power of the ULS labelling technology is its ability to label any and all sizes of naturally occurring nucleic acids witho enzymes.

Kreatech's ULS technology relies on the binding properties of platinum, to form coordinative bonds with biomolecule specific sites on DNA, RNA and proteins.

In this way, ULS acts as "molecular glue" for DNA, RNA and proteins. As a result of its versatility, customers have a molecular label attached to the ULS they can use to label the biomolecules of interest.

AIDS Research and Therapy

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HIV-1 resistance conferred by siRNA cosuppression of CXCR4 and CCR5 coreceptors by a bispecific lentiviral vector

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Abstract

Background: RNA interference (RNAi) mediated by small interfering RNAs (siRNAs) has proved to be a highly effective gene silencing mechanism with great potential for HIV/AIDS gene therapy. Previous work with siRNAs against cellular coreceptors CXCR4 and CCR5 had shown that down regulation of these surface molecules could prevent HIV-1 entry and confer viral resistance. Since monospecific siRNAs targeting individual coreceptors are inadequate in protecting against both T cell tropic (X4) and monocyte tropic (R5) viral strains simultaneously, bispecific constructs with dual specificity are required. For effective long range therapy, the bispecific constructs need to be stably transduced into HIV-1 target cells via integrating viral vectors.

Results: To achieve this goal, lentiviral vectors incorporating both CXCR4 and CCR5 siRNAs of short hairpin design were constructed. The CXCR4 siRNA was driven by a U6 promoter whereas the CCR5 siRNA was driven by an H1 promoter. A CMV promoter driven EGFP reporter gene is also incorporated in the bispecific construct. High efficiency transduction into coreceptor expressing Magi and Ghost cell lines with a concomitant down regulation of respective coreceptors was achieved with lentiviral vectors. When the siRNA expressing transduced cells were challenged with X4 and R5 tropic HIV-1, they demonstrated marked viral resistance. HIV-1 resistance was also observed in bispecific lentiviral vector transduced primary PBMCs.

Conclusions: Both CXCR4 and CCR5 coreceptors could be simultaneously targeted for down regulation by a single combinatorial lentiviral vector incorporating respective anti-coreceptor siRNAs. Stable down regulation of both the coreceptors protects cells against infection by both X4 and R5 tropic HIV-1. Stable down regulation of cellular molecules that aid in HIV-1 infection will be an effective strategy for long range HIV gene therapy.

Background

HIV/AIDS continues to be a major public health problem worldwide with millions of people currently infected and new infections being on the rise. As no effective vaccines are currently available for prevention, new and innovative therapies need to be developed. Although combinatorial therapies such as HAART have proven to be effective in prolonging life, they do not afford a complete cure. Other

constraints with HAART therapy are the development of drug resistant viral mutants and toxicity after prolonged therapy. Intracellular immunization by gene therapy strategies offers a promising alternative approach for controlling and managing HIV disease. A number of previous approaches that involved the use of transdominant proteins [1-3], decoys [3-7], and ribozymes [5,8-12] had shown initial promise but fell short of practical utility in providing adequate protection. With the discovery that the RNA interference phenomenon operates in mammalian cells and is highly effective in selective gene silencing, new potent small interfering RNA (siRNA) molecules have become available to add to the anti-HIV arsenal [13].

RNAi is a highly potent mechanism of post-transcriptional gene silencing. Mediated by sequence specific siRNAs, it can effectively down regulate expression of either viral or cellular RNA target molecules by selective degradation of mRNAs [13-16]. Mechanism of destruction involves an endonuclease present in the RISC complex which is guided by the antisense component of the siRNA for target recognition. A number of reports have shown that delivery of siRNAs by transfection of presynthesized or plasmids encoding siRNAs into cultured cells can effectively inhibit HIV-1 infections [17-26]. Antiviral effects of these delivery methods are only transient due to eventual degradation and dilution of siRNAs during cell division. For HIV gene therapy strategies to succeed in long range, it is necessary that siRNA coding transgenes be maintained and expressed long term in a virus susceptible target cell. In this regard, lentiviral vectors have proven to be highly effective in high efficiency gene transduction and sustained gene expression.

A number of previous approaches using either synthetic siRNAs or plasmid expressed constructs have successfully targeted viral transcripts and achieved effective viral inhibition. Of these, some anti-HIV-1 siRNAs, such as siRNAs against tat, tat-rev had been introduced into lentiviral vectors and their efficacy was demonstrated both in cell lines and primary T cells and macrophages [27,28]. Promising data was also obtained in experiments showing that anti-rev siRNAs against HIV-1 were functional in conferring viral resistance in differentiated T cells and macrophages derived from lentiviral transduced CD34+ hematopoietic progenitor cells [29].

In addition to targeting viral transcripts, many studies including ours also investigated the efficacy of siRNAs in down regulating host cell molecules necessary for HIV-1 infection [18,21,23,24,30,31]. An advantage in targeting cellular molecules is that efficacy will be more broad spectrum against all the clades of the virus and the frequency of escape mutants will be lower. Down regulation of the primary cell surface receptor CD4 and consequent inhibition of HIV-1 infection was shown using synthetic siRNAs. However, since CD4 is an essential cell surface molecule for immunological function, it is not a practical target for HIV gene therapy. Chemokine receptors CCR5 and CXCR4 play critical roles as coreceptors for viral entry during infection with macrophage tropic R5 and T cell tropic X4 HIV-1 viral strains respectively [32,33]. Thus they are suitable targets for siRNA mediated down regulation. Since both R5 and X4 viral strains are involved in disease pathogenesis, it is important to consider blocking of both respective coreceptors when developing effective therapeutics. In a segment of the human population, a naturally occurring 32-bp deletion in the CCR5 gene results in the loss of this coreceptor thus conferring significant resistance to HIV infection [34-36]. Homozygous or heterozygous individuals for this mutation remain physiologically normal. With regard to the CXCR4 coreceptor, it was found to be dispensable for T cell development and maturation in murine studies [37]. These findings suggest that CCR5 and CXCR4 are promising targets for HIV therapies.

Based on this rationale, recent work with synthetic siRNAs demonstrated that down regulating either CXCR4 or CCR5 will protect cells from X4 or R5 HIV-1 strains respectively at the level of viral entry [18,21,23,24]. Although stable expression of an anti-CCR5 siRNA was achieved using a lentiviral vector in one study, down regulating CCR5 alone in the face of an HIV-1 infection is insufficient [31]. Therefore, we recently experimented with synthetic bispecific combinatorial constructs targeted to both CXCR4 and CCR5 and have shown their efficacy in cultured cells [24]. To make further progress, our present studies are directed towards constructing a single bispecific lentiviral vector expressing both CXCR4 and CCR5 siRNAs. Using this combinatorial construct, here we show high efficiency transduction, simultaneous down regulation of both coreceptors resulting in HIV-1 resistance.

Results and Discussion

Coreceptor down regulation by a bispecific lentiviral vector

Our major goal in these studies is to introduce both CXCR4 and CCR5 siRNAs into a single lentiviral construct to achieve their stable expression in transduced cells. Lentiviral vectors offer advantages over conventional retroviral vector systems since they can transduce dividing as well as nondividing cells and are less prone to transgene silencing [44-47]. The transfer vector HIV-7-GFP-XHR (referred to as XHR) contained a short hairpin type anti-CXCR4 siRNA driven by a Pol-III U6 promoter followed by a short hairpin anti-CCR5 siRNA driven by a different Pol-III promoter, H1. Downstream, the reporter gene, EGFP is driven

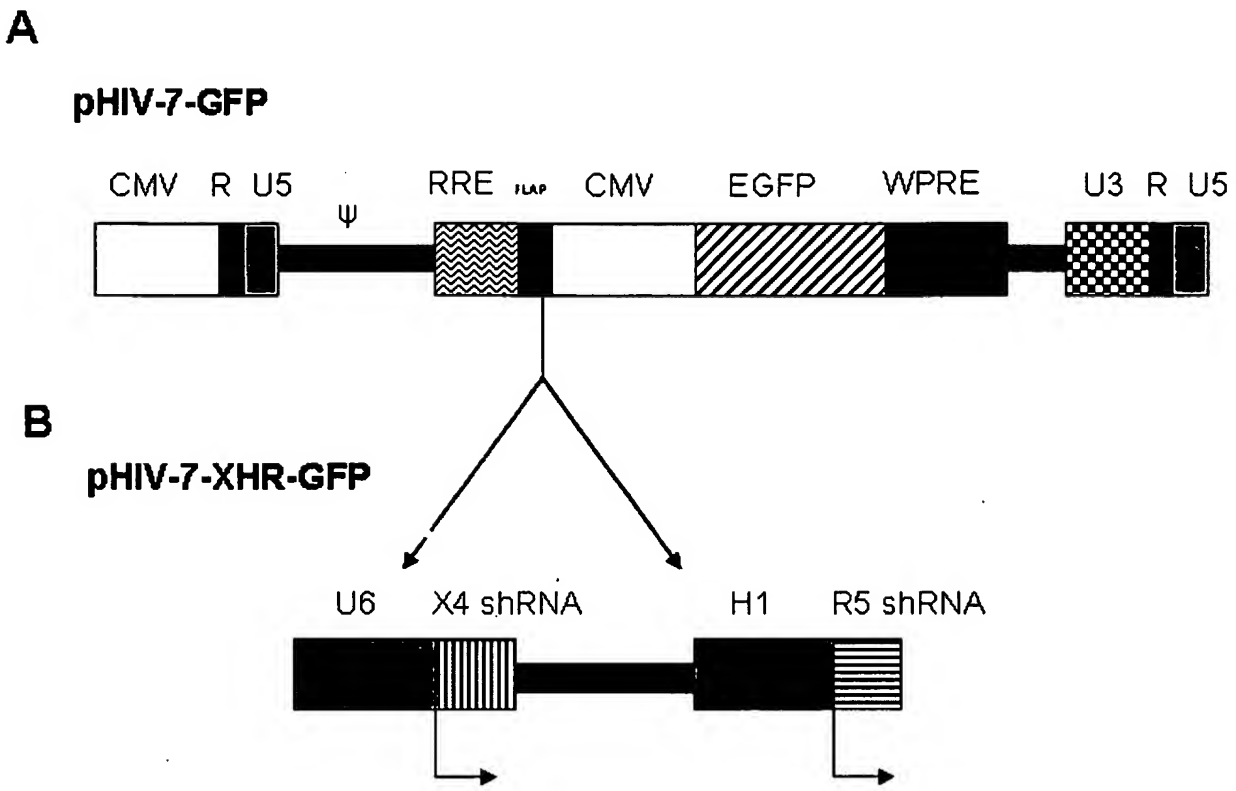

Figure 1
Bispecific lentiviral vector (XHR) encoding anti-CXCR4 and CCR5 siRNAs. A) Control transfer vector pHIV-7-GFP encoding a CMV promoter driven EGFP reporter gene. B) To derive the bispecific vector pHIV-XHR-GFP, a U6 promoter driven short hairpin CXCR4 siRNA cassette was cloned into the *BamHI* site upstream to the CMV-EGFP cassette. The H1-CCR5 siRNA cassette was inserted into an *MluI* site downstream to the U6-CXCR4 siRNA cassette.

by a CMV promoter. The control GFP-alone vector, HIV-7-GFP, contained only the reporter gene EGFP (Fig 1).

Magi-CXCR4 cells constitutively expressing CXCR4 on the cell surface when transduced with the control vector or XHR vector had shown 97% and 83% EGFP expression respectively as measured by FACS analysis indicating high efficiency of transduction (Fig 2A and 2C). To determine if CXCR4 was down regulated by the respective siRNA in the XHR construct, the transduced cells were analyzed for CXCR4 surface expression. The surface levels of CXCR4 were reduced significantly in XHR transduced cells (73% lower) compared to the cells transduced with control vector (Fig 2B and 2D) indicating the efficacy of the CXCR4 siRNA on its target. Similarly, to determine the activity of the anti-CCR5 siRNA in the XHR vector, transduced Ghost R5 cells that constitutively express CCR5 were evaluated. As seen in Fig 3A and 3C, high levels of transduction (84% and 83%) were seen in Ghost-R5 cells with either the control vector or XHR vector, respectively. When the transduced cells were analyzed for CCR5 expression, a dramatic decrease in CCR5 expression was seen in XHR cells (72%) compared to control vector transduced cells (Fig 3B and 3D). These results had shown that the bispecific lentiviral vector XHR efficiently down regulates both CXCR4 and CCR5 targets in respective cells.

Expression of siRNAs and down regulation of CXCR4 and CCR5 transcripts

To confirm that the down regulation of both CXCR4 and CCR5 coreceptors as seen by FACS analysis is due to reduced levels of the corresponding mRNAs, vector transduced cells were analyzed by RT-PCR. As an internal control, GAPDH mRNA was also analyzed. XHR vector transduced cells showed considerable reduction in transcript levels for both CXCR4 and CCR5 as compared to

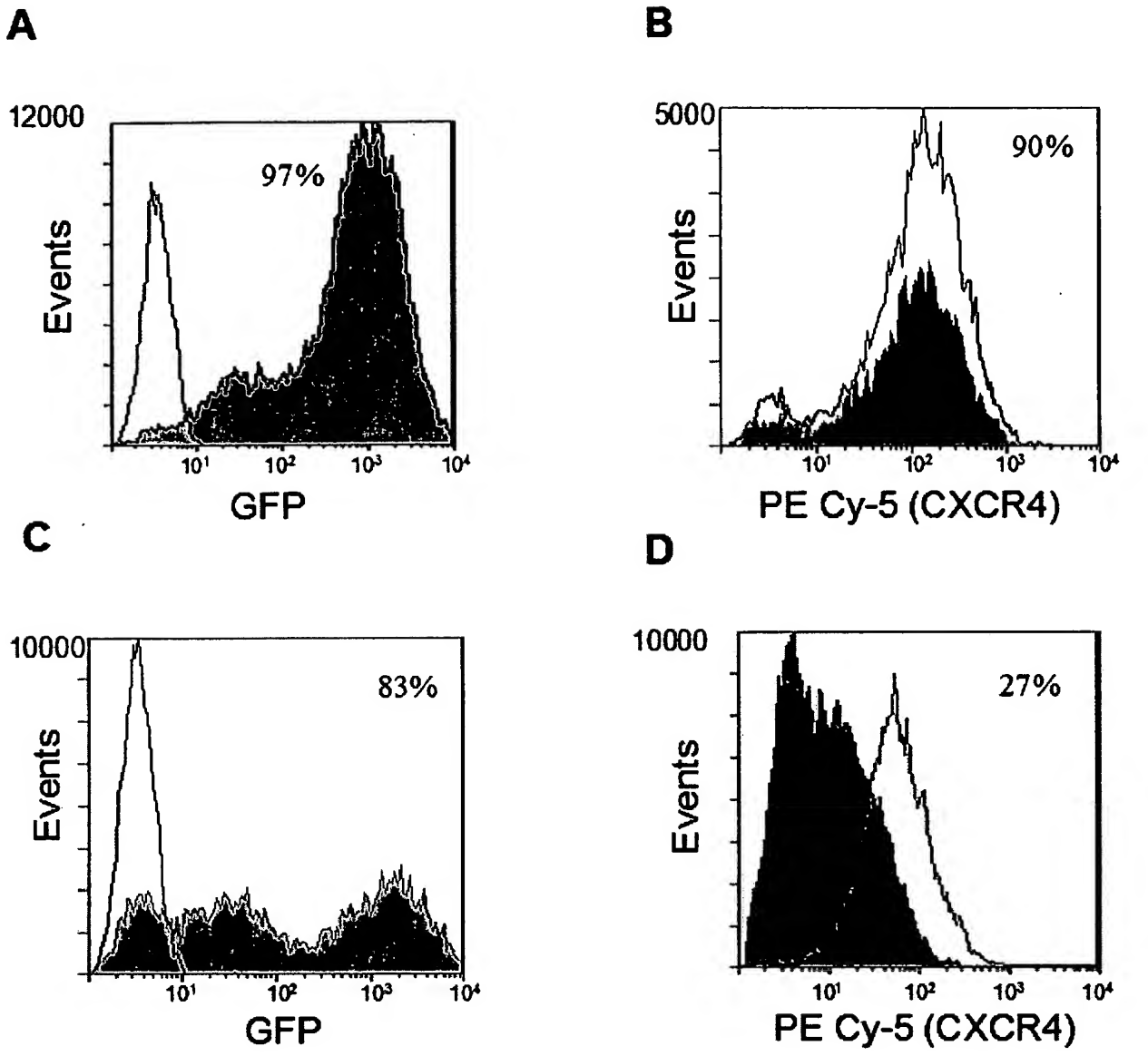

Figure 2
Cell surface down regulation of CXCR4 in XHR transduced Magi-CXCR4 cells. Magi-CXCR4 cells that constitutively express CXCR4 were transduced with control GFP or XHR vectors. Cells were stained with PECy5-conjugated antibodies to CXCR4 and analyzed by FACS 72 hours post-transduction. Levels of CXCR4 in non-transduced cells are superimposed (unshaded areas). Transduction efficiency was determined by FACS for EGFP expression. Levels of EGFP in control GFP-alone vector (A) and XHR vector (C) transduced cells. Levels of CXCR4 expression in GFP-alone (B) and XHR (D) vector transduced cells. Percent positive cells are indicated.

non-transduced and control GFP vector transduced cells. The levels of GAPDH control mRNA remained unchanged in all samples (Fig 4). To validate the expression of individual siRNAs in transduced Magi-CXCR4 and Ghost R5 cells, cellular RNA was analyzed by northern analysis for their presence. As internal controls, the presence of constitutively expressed miRNA-16 RNAs were also analyzed in parallel. As expected, comparable levels of miRNA-16

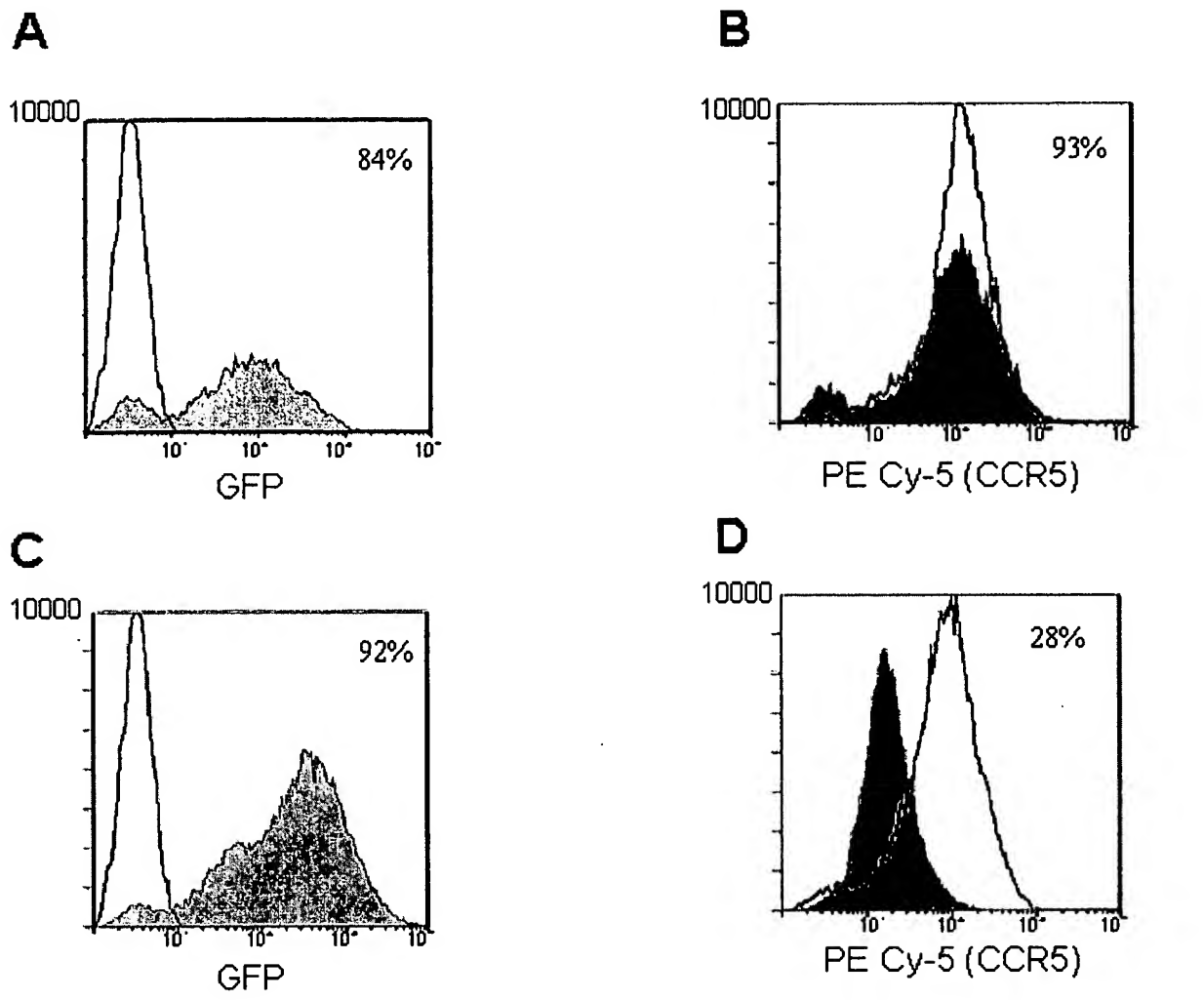

Figure 3
Cell surface down regulation of CCR5 in XHR transduced Ghost-R5 cells. Ghost-R5 cells that constitutively express CCR5 were transduced with GFP-alone or XHR vectors. Cells were stained with PECy5-conjugated antibodies to CCR5 and analyzed by FACS 72 hours post-transduction. Levels of CCR5 in non-transduced cells are superimposed (unshaded areas). Transduction efficiency was measured by FACS for EGFP expression. Levels of EGFP in control GFP-alone vector (A) and XHR vector (C) transduced cells. Levels of CCR5 expression in GFP-alone (B) and XHR (D) vector transduced cells. Percent positive cells are indicated.

RNAs (22 bp in length)were detected in GFP control vector transduced as well as in XHR vector transduced cells (Fig 5A). RNAs corresponding to CXCR4 and CCR5 shRNAs (representing the 21nt antisense strand of each shRNA) were seen in XHR transduced but not in GFP control vector transduced cells (Fig 5B).

Bispecific siRNA vector does not induce interferon

Double stranded RNA molecules longer than ~30 bp are known to induce the interferon pathway in response to viral infections. As siRNAs are generally comprised of 19–24 bp in length, they are not expected to activate such a response that mediates a non-specific down regulation of

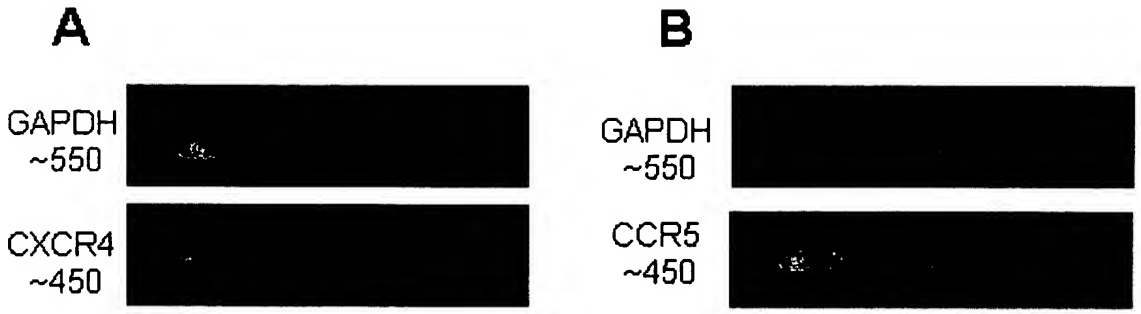

Figure 4
RT-PCR detection of CXCR4 and CCR5 mRNA down regulation. Total RNA was extracted from vector transduced cells and one-step RT-PCR was performed. PCR products of 450 bp were amplified to detect the coreceptor transcripts. A) Levels of CXCR4 mRNA in non-transduced (lane 1), GFP-alone (lane 2), and XHR (lane 3) vector transduced Magi-X4 cells. B) CCR5 transcript levels in non-transduced (lane 1), GFP-alone (lane 2), and XHR vector transduced Ghost-R5 cells. GAPDH transcript levels were used as internal controls (PCR product size ~550 bp).

cellular or viral mRNAs. However, recent data had shown that in some circumstances, certain siRNAs might induce variable levels of interferon activation [48-50]. To rule out such a possibility with the present siRNAs, we looked for upregulation of phosphorylated-PKR by western blot analysis. PKR is a protein kinase that becomes activated through phosphorylation in the presence of dsRNA and is involved during the interferon response. Our results have shown that the levels of phosphorylated PKR remain unchanged in XHR transduced cells similar to mock and GFP vector transduced cells. In contrast, elevated levels of phosphorylated PKR could be seen in poly I:C transfected cells used as positive controls (Fig 6). These data exclude the possibility of non-specific interferon activation by the combinatorial lentiviral construct.

Resistance of siRNA transduced cells to HIV-1 infection

To determine if down regulation of the essential coreceptors, CXCR4 and CCR5, translated to virus resistance, transduced Magi-CXCR4 and Ghost R5 cells were challenged with X4 (NL4-3) and R5 (BaL1)-tropic strains of HIV-1 respectively. Viral p24 antigen levels at different days post-challenge were determined by ELISA to quantify levels of HIV-1 resistance. Over a 10-fold reduction in viral antigen levels was seen with both XHR transduced Magi-CXCR4 and Ghost-R5 cells as compared to non-transduced and GFP-alone vector transduced cells (Fig 7). There was a slight increase in viral production in XHR transduced cells on days 5 to 7. This could be due to non-transduced and/or low siRNA expressing cells producing the virus. We next wanted to determine if the XHR vector expressing CXCR4 and CCR5 siRNAs is effective in physiologically relevant cells for gene therapy. Accordingly, PBMCs transduced with vectors were challenged in the same manner as above. A 3-fold level of inhibition was seen on days 3, 5, and 7 (Fig 8). These results established that the XHR vector is also effective in primary cells in inhibiting HIV-1. Although clearly significant, the levels of virus inhibition were not as dramatic as seen with Magi and Ghost cell lines. The observed levels of viral inhibition in primary PBMC are similar to those observed in a recent report [31]. Lower levels of protection in PBMCs were likely due to the lower levels of transduction. Future studies that are aimed at increasing transduction efficiencies into primary lymphocytes and macrophages are likely to overcome this hurdle.

In summary, our studies have shown for the first time that a single lentiviral vector could be used to stably deliver two different siRNAs targeted to two different cell surface co-receptor molecules and achieve protection against both X4 and R5 tropic HIV-1 viral strains. The short hairpin design permitted use of a single promoter to transcribe both the sense and anti-sense strands of each of the siRNAs. No promoter interference was observed between the U6 promoter driving the transcription of CXCR4

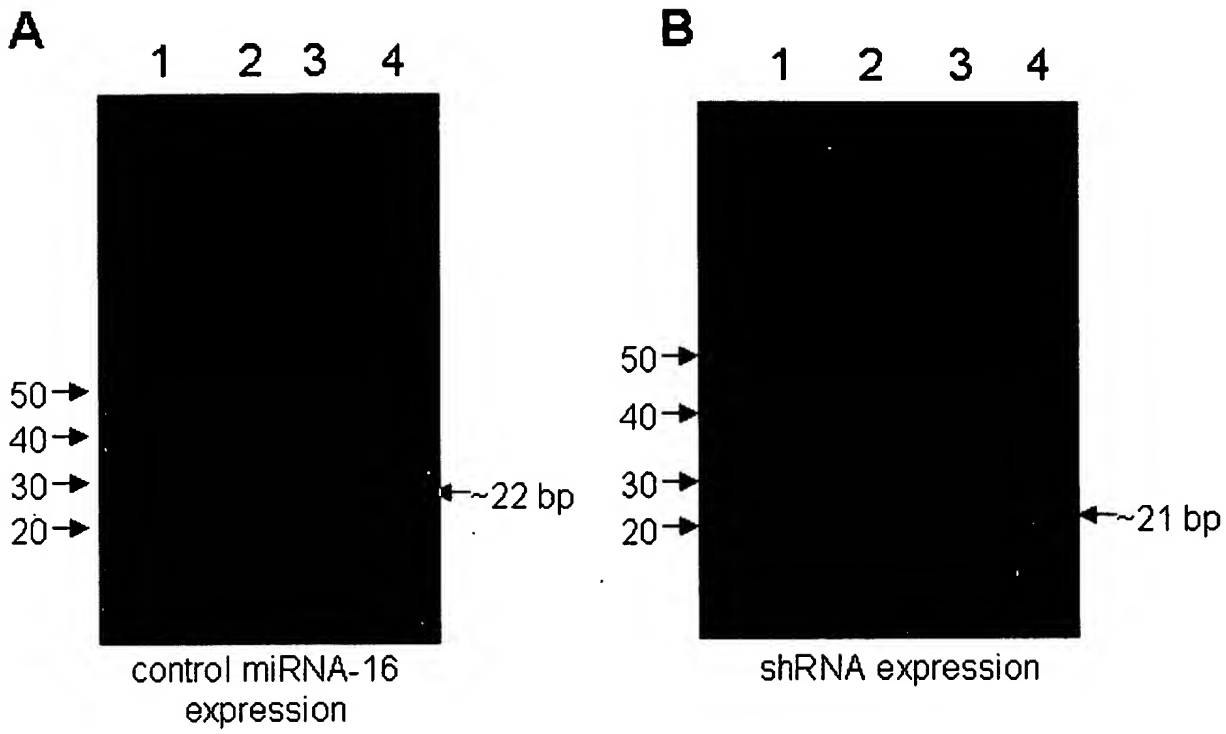

Figure 5
Northern analysis to detect siRNA expression in transduced cells. Small RNAs (<200 nt) were extracted from transduced cells and probed with specific primers to detect the expression of siRNAs as described in materials and methods. A) Northern blot to detect the presence of miRNA-16 (~22 bp) as an internal control in GFP-alone vector transduced (lane 2) and XHR transduced Magi-X4 (lane 3) and Ghost-R5 (lane 4) cells. B) siRNA (~21 bp) detection in GFP-alone vector transduced (lane 2) and XHR transduced Magi-X4 (lane 3) and Ghost-R5 (lane 4) cells. Decade markers (lanes A1 and B1).

siRNA and the H1 promoter driving the CCR5 siRNA since comparable amounts of both the siRNAs could be seen in transduced cells. Furthermore, possible interferon induction by the combinatorial construct was also ruled out.

A major advantage in using a combinatorial lentiviral construct targeted to both the coreceptors is that infection with either of the viral strains could be prevented at the entry step thus eliminating the possibility of proviral integration and viral latency. Given the success with the current bispecific construct, other novel constructs could be designed and experimented with that incorporate siRNAs targeted to both the cellular as well as viral targets. Based on the design employed here, it is possible to introduce more than two siRNAs in a single construct in the future. However caution should be exercised while incorporating multiple siRNAs in a single construct because the possibility exists that over expression of foreign siRNAs in a cell may have undesirable effects such as saturating the endogenous RISC complex and consequent toxicity. Such a possibility needs to be tested in long range experiments *in vivo*. We previously have introduced a monospecific siRNA targeted to HIV-1 *rev* into CD34 hematopoietic progenitor cells via lentiviral vectors and derived transgenic macrophages *in vitro* and T cells *in vivo* [29]. The transgenic cells were found to be apparently normal while markedly resistant to HIV-1 infection.

No deleterious effects are expected by the stable knock down of the CCR5 coreceptor *in vivo* since individuals harboring a 32 bp deletion in the corresponding gene are physiologically normal [34,35]. Although CXCR4 down regulation in circulating mature T cells in the periphery may not have any insurmountable ill effects, this may have possible drawbacks in a stem cell setting due to its role in cell homing into bone marrow [51,52]. Additionally, recent gene expression profiling studies indicated

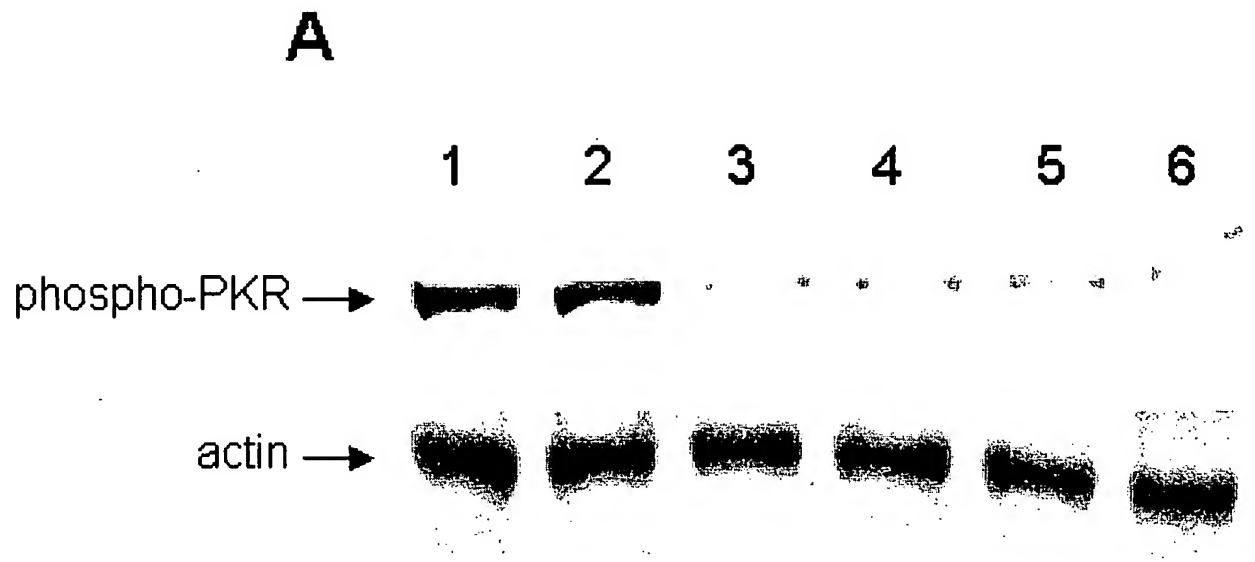

Figure 6
Lack of interferon induction in siRNA transduced cells. To detect interferon induction in siRNA vector transduced cells, western blot analysis was performed to detect elevated levels of phophorylated PKR. Poly I:C was used to induce interferon as a positive control. Transduced cell extracts were run on 10% SDS-PAGE gels, transferred, and probed with an anti-phospho-PKR antibody. Positive control poly I:C transfected (lanes 1 and 2), non-transduced (lane 3), GFP-alone vector (lane 4), and XHR transduced (lane 5) Magi-X4 cells and XHR transduced Ghost-R5 cells (lane 6). An anti-actin antibody was used as an internal control.

some off-target effects by siRNAs [53]. Therefore, the present combinatorial construct targeted to both CXCR4 and CCR5 coreceptor molecules need to be thoroughly tested in an *in vivo* system such as the SCID-hu mouse model to evaluate its efficacy and possible toxicity in differentiated cells before it can be used for gene therapy in human subjects. Such experiments are currently underway.

Conclusions

For HIV/AIDS gene therapy strategies to succeed, novel molecules need to be harnessed. In this regard, siRNAs offer great potential. Exploitation of these promising candidates to down regulate essential cellular coreceptors via the use of lentiviral vectors facilitates long term derivation of resistant T cells and macrophages which are the main targets for the virus. Our results showed for the first time that expression of both CXCR4 and CCR5 siRNAs in combination is possible by the use of lentiviral vectors. Coreceptor specific siRNAs stably transduced with the bispecific lentiviral vector showed marked resistance against both T cell tropic and monocyte tropic HIV-1 infection in cell lines and primary PBMCs. The newly developed bispecific vector shows promise for potential *in vivo* application.

Materials and Methods

Plasmid and lentiviral vector construction

Previously characterized siRNAs against CXCR4 and CCR5 were used in generating the bispecific lentiviral vector [23,24,30]. A third generation lentiviral vector backbone was employed to derive the bispecific constructs. The two *cis*-acting elements, namely, the central DNA flap consisting of cPPT and CTS (to facilitate the nuclear import of the viral preintegration complex) and the WPRE (to promote nuclear export of transcripts and/or increase the efficiency of polyadenylation of transcripts), are engineered to enhance the performance of the vector [38,39]. An siRNA expression cassette targeting CXCR4 under the control of the Pol-III U6 promoter was PCR amplified from the plasmid pTZ-U6+1 as described by Castanotto *et al* [40]. This cassette was cloned into pHIV-7-GFP transfer vector in the *BamHI* site immediately upstream of the CMV-EGFP gene. This cassette contained a *MluI* restriction site downstream from the CXCR4 siRNA sequence for subsequent cloning of the H1 promoter driven CCR5

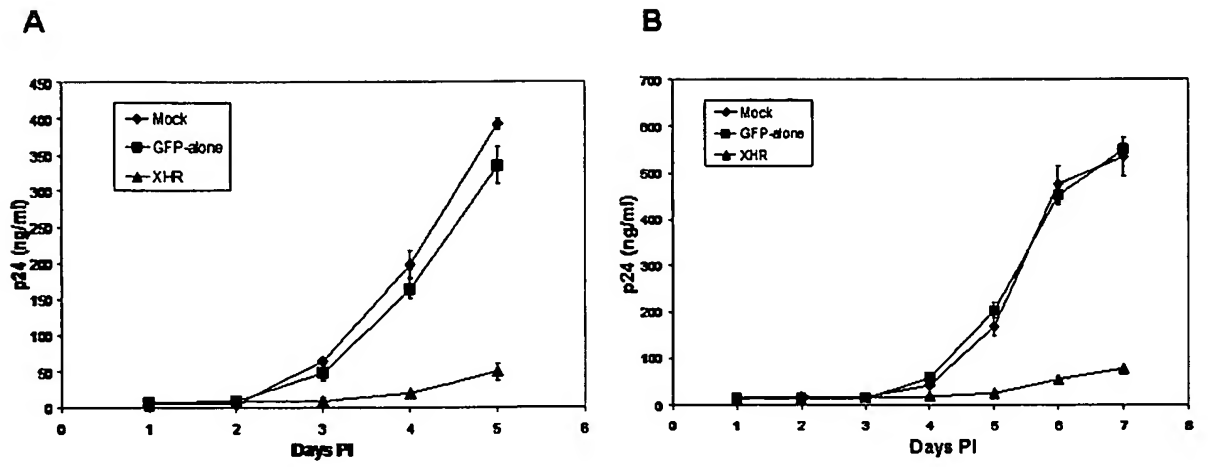

Figure 7
HIV-1 challenge of XHR transduced Magi-X4 and Ghost-R5 cells. Vector transduced cells were challenged with either X4 tropic or R5 tropic viruses at an m.o.i of 0.01. Culture supernatants were collected at different days post challenge and p24 antigen was assayed by ELISA. A) Transduced Magi-X4 cells challenged with X4 tropic HIV-1 NL4-3. B) Transduced Ghost-R5 cells challenged with R5 tropic HIV-1 BaL-1. Data presented is from triplicate experiments.

siRNA cassette. The H1-CCR5 siRNA expression cassette was also generated as described above using the plasmid pSUPER (Oligoengine, Seattle, WA). Sequencing and confirmation of candidate clones was performed by Laragen Inc. (Los Angeles, CA). The transfer vector containing the inserts U6-X4 siRNA and H1-CCR5 siRNA is termed pHIV-XHR-GFP.

Cell culture and vector production

293T cells and PBMCs were maintained in DMEM media supplemented with 10% FBS. Magi-CXCR4 cells obtained from the AIDS Reference and Reagent Program were maintained in media as previously described [41,42]. Ghost-R5 cells obtained from the AIDS Reference and Reagent Program were maintained in media as previously described [43]. To generate lentiviral vectors, fifteen micrograms of transfer vector with either GFP-alone or XHR were transfected along with 15 ug pCHGP-2, 5 ug pCMV-Rev, and 5 ug pCMV-VSVG into 293T cells at 60% confluency in 100 mm culture dishes using a calcium phosphate transfection kit (Sigma-Aldrich, St. Louis, MO). Six hours after transfection, fresh medium was exchanged. Cell culture supernatants containing the vector were collected at 24, 36, 48, and 60 hours post transfection and pooled. Vector supernatants were concentrated by ultracentrifugation and later titrated on 293T cells using FACS analysis for GFP expression.

Lentiviral vector transduction and FACS analysis

Magi-CXCR4 and Ghost-CCR5 cells were seeded in 6-well plates 24 hours prior to transduction, 5×10^5 cells per well. Cells were transduced with lentiviral vectors at an m.o.i. of 10 in the presence of 4 ug/ml polybrene for 2 hours. For transduction of PBMCs, cells were first isolated from whole blood by Histopaque®-1077 (Sigma-Aldrich), and then cultured in CD3 and CD28 antibody coated plates. Three days after stimulation, PBMCs were transduced at an m.o.i of 20 in the presence of 4 ug/ml polybrene. PBMC transduction was repeated the following day. Seventy-two hours post transduction with siRNA containing lentiviral vectors, FACS analysis was performed to determine the levels of cell surface expression of CXCR4 and CCR5. Non-transduced and transduced cells were stained with appropriate antibodies conjugated with PE-Cy 5 (Pharmingen, San Diego, CA) namely, anti-CXCR4 for Magi-CXCR4 cells and anti-CCR5 for Ghost-CCR5 cells. Transduction efficiency was determined by assaying for EGFP expression. FACS analysis was performed on the Beckman Coulter Epics XL using ADC software for analysis.

Northern analysis for shRNA expression

Total RNA was extracted from non-transduced and transduced Magi-CXCR4 and Ghost-CCR5 cells using the RNA-STAT-60 reagent (Tel-Test, Friendswood, TX). Small

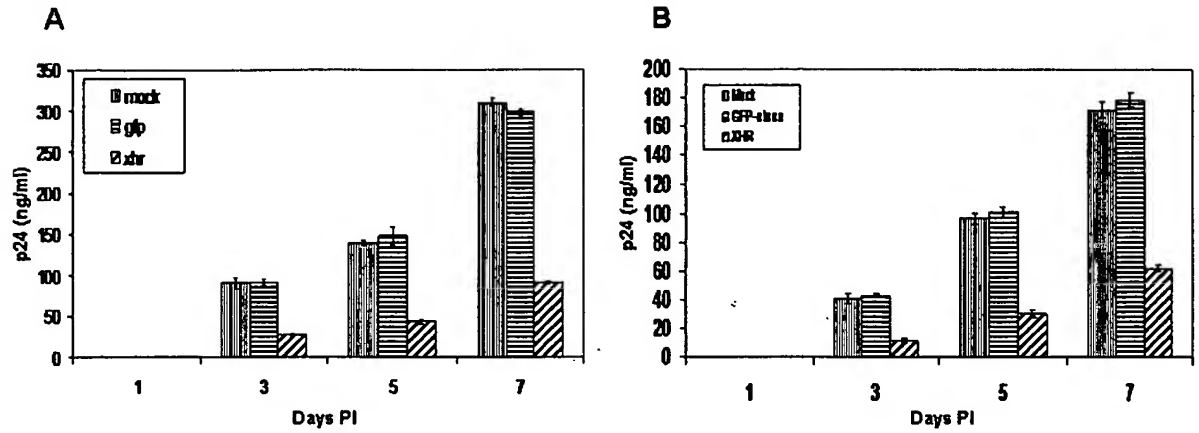

Figure 8
HIV-1 challenge of XHR transduced PBMCs. Vector transduced PBMCs were challenged with either X4 tropic or R5 tropic viruses. Culture supernatants were collected at different days post challenge and p24 antigen was assayed by ELISA. Transduced PBMCs challenged with either HIV-1 NL4-3 (A) or BaL-1 (B). Data presented is from triplicate experiments.

RNAs, <200 nt, were separated and concentrated using the *mir*Vana™ miRNA Isolation Kit (Ambion, Austin, TX). Twenty micrograms of small RNAs were hybridized overnight at 37°C using the *mir*Vana™ miRNA Detection Kit (Ambion) with γ-^{32}P labeled probes made using the *mir*Vana™ Probe & Marker Kit (Ambion). Probes were complementary to the antisense strands of CXCR4 and CCR5 siRNAs. Hybridization reactions were processed according to the manufacturer's protocol and run on 15% polyacrylamide TBE-Urea gels. Gels were then exposed to X-ray film. A probe complementary to miRNA-16 supplied with the miRNA detection kit was used as an internal control.

Western Blot analysis of phosphorylated PKR

Cell lysates of non-transduced and transduced cells were run on 10%-polyacrylamide-SDS TBE gels. Proteins were immunoblotted onto Immobilon™-P membranes (Millipore, Bedford, MA) and incubated with antibody specific for phosphorylated-PKR (Sigma-Aldrich), while anti-actin antibody (Sigma-Aldrich) was used to detect cellular actin as an internal control. A secondary antibody, goat anti-rabbit IgG conjugated with alkaline phophatase (Promega, Madison, WI), was then added. An alkaline phophatase substrate reagent, Western Blue (Promega), was used to visualize the bands.

RT-PCR

Total RNA was extracted from non-transduced and transduced cells. Primers specific for CXCR4 (forward: 5'-ggaggggatcagtatatacacttc and reverse: 5'-cgccaacatagaccacctttc) and CCR5 (forward: 5'-caaaaagaaggtcttcattacacc and reverse: 5'-cttgctcgctcgggagcctc) (IDT, Coralsville, IA) were used to determine transcript levels while GAPDH (forward: 5'-ctgagaacgggaagcttgtcatcaa and reverse: 5'-gcctgcttcaccaccttcttgatg) primers were used as an internal control. One-step RT-PCR reactions were performed using the Superscript™ III One-Step RT-PCR kit (Invitrogen, Carlsbad, CA). Reactions were run on 1% agarose gels and appropriate bands were visualized with UV light.

HIV-1 Challenge

To determine if down-regulation of CXCR4 and CCR5 transcript levels and cell surface expression inhibited HIV-1 infection, non-transduced and transduced cells were challenged with NL4-3 (X4-tropic) and BaL-1 (R5-tropic) strains of HIV-1, at an m.o.i of 0.01, as previously described [24]. Viral supernatants were collected daily

from infected Magi-CXCR4 and Ghost-CCR5 cells for p24 assay. ELISA was used to determine p24 values employing a Coulter-p24 kit (Beckman Coulter, Fullerton, CA). For PBMC challenge experiments, non-transduced and transduced cells were infected with NL4-3 and Bal-1 strains and cell culture supernatants were collected on days 1, 3, 5, and 7 post-infection to measure p24 levels.

Competing interests

The author(s) declare that they have no competing interests.

Author's contributions

JA carried out all of the experiments. RA was responsible for the overall experimental design and implementation of the project.

Acknowledgements

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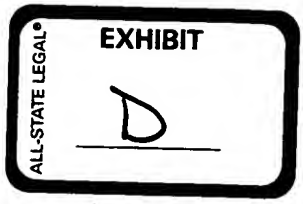

Protocols

A Simple and Rapid Method to Detect Plant siRNAs Using Nonradioactive Probes

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Abstract. Small interfering RNAs (siRNAs) are key molecules in RNA silencing, which includes posttranscriptional gene silencing, cosuppression, quelling, and RNA interference. The presence of siRNAs indicates RNA silencing in cells. We present a method of detecting siRNAs using nonradioactive probes that involves isolating the small RNA fraction, separating siRNAs using denaturing gel electrophoresis, and performing a Northern blot analysis under low-stringency hybridization conditions. We used digoxigenin-labeled DNA probes for hybridization and detected siRNAs in petunia and rice plants exhibiting silenced phenotypes. This method is a simple and rapid way to detect siRNAs without using radioisotopes.

Key words: digoxigenin, nonradioactive probes, Northern blot analysis, PTGS, RNA silencing, siRNAs

Abbreviations: CaMV, cauliflower mosaic virus; CHS-A, chalcone synthase-A; NOS, nopaline synthase; PAGE, polyacrylamide gel electrophoresis; PEG, polyethylene glycol; PTGS, posttranscriptional gene silencing; RNAi, RNA interferernce; siRNAs, small interfering RNAs.

Introduction

RNA silencing is gene silencing involving a sequence-specific degradation of RNA. It includes posttranscriptional gene silencing (PTGS), cosuppression, and RNA-mediated virus resistance in plants; RNA interference (RNAi) in animals; and silencing in fungi (quelling in *Neurospora*) and algae (Matzke et al., 2001; Plasterk, 2002). These systems are mechanistically related, as the required genes show sequence homology (Matzke et al., 2001), and small RNA molecules (21-26 nt) complementary to both strands of the silenced genes are found (Plasterk, 2002; Zamore, 2002). Small RNA molecules, known as small interfering RNAs (siRNAs), were first discovered in transgenic plants exhibiting PTGS (Hamilton and Baulcombe, 1999). Biochemical experiments with cytoplasmic extracts from *Drosophila* revealed that siRNAs are produced by processing dsRNA

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by RNaseIII-like enzymes (Tuschl et al., 1999; Zamore et al., 2000; Elbashir et al., 2001; Bernstein et al., 2001). siRNAs direct mRNA cleavage by sequence complementarity (Hammond et al., 2000); thus, the presence of siRNAs indicates the occurrence of RNA silencing in cells.

siRNAs can be detected by means of Northern blot analysis. The original method of detecting siRNAs involves isolating nucleic acids from tissues, enriching the small RNA fraction, size-fractionating the small RNA using denaturing polyacrylamide gel electrophoresis (PAGE), blotting the RNA to a membrane, hybridizing the RNA on the membrane with a labeled probe, and detecting the hybridization signal (Hamilton and Baulcombe, 1999). Probes labeled with radioisotope are prepared by in vitro transcription from plasmid DNA. Labeled RNAs are then hydrolyzed by sodium bicarbonate treatment to obtain probes averaging 50 nt before hybridization (Hamilton and Baulcombe, 1999). The use and disposal of radioisotopes in this process is costly, labor intensive, and hazardous. Therefore, we developed a nonradioactive protocol to detect siRNAs using a digoxigenin system for nucleic acid detection. Our method is quick and simple and does not require radioisotopes. It can be used to detect siRNAs in various PTGS and RNAi systems in different organisms.

Materials and Methods

Plant materials

Wild-type petunia (*Petunia hybrida*) V26 line and a transgenic line of V26 (C001; O'Dell et al., 1999) with petunia chalcone synthase-A (CHS-A) gene were used. The transgenic petunia plant expresses CHS-A transgene under the control of the cauliflower mosaic virus (CaMV) 35S promoter and nopaline synthase (NOS) terminator. Gene silencing is observed as changes in flower color because CHS-A catalyses an essential step in the biosynthesis of anthocyanin pigments. Wild-type and silenced plants produce purple and white flowers, respectively (Figure 1) (Metzlaff et al., 1997, 2000; O'Dell et al., 1999; Kanazawa et al., 2000). A rice (*Oryza sativa*) strain exhibiting the PTGS phenotype for *glutelin* genes was also used (M. Kusaba, K. Miyahara, S. Iida, H. Fukuoka, T. Takano, H. Sassa, M. Nishimura, and T. Nishio, submitted). Nucleic acids were extracted from petal tissues of 4 petunia buds (30-45 mm) and 30 developing rice seeds.

Hybridization probes

We used a petunia CHS-A gene probe, a soybean 5S RNA probe, and a rice *glutelin* gene probe. The CHS-A gene probe was prepared by amplifying a 0.44-kb sequence corresponding to the 3' end of exon 2 from petunia CHS-A cDNA by using specific primers '4246' (5'-GGCGCGATCATTATAGGTTC-3') and '2349' (5'-CCCTTCACCAGTAGTTCCTA-3'). For the 5S RNA probe, a 0.25-kb sequence of the 5S RNA gene was amplified by PCR from genomic soybean DNA (*Glycine max* cv. 'Harosoy') by using specific primers 'SOJA 5S-1' (5'-GAAGTCCTCGTGTTGCACC-3') and 'SOJA 5S-2' (5'-AGTGCTGGTATG-ATCGCACT-3'). The amplified fragment was used as a template for PCR labeling using the same primers. For the *glutelin* gene probe, a *glutelin* cDNA clone

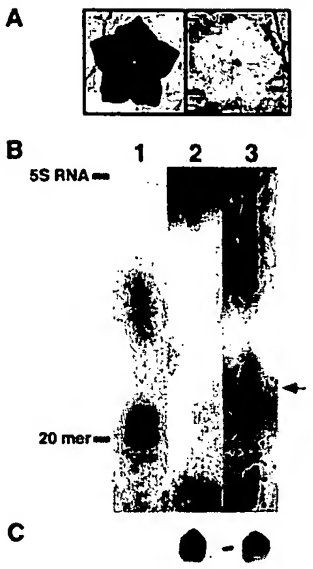

Figure 1. Northern blot analysis of low molecular weight RNA from wild-type and transgenic petunia. (A) Flower phenotypes of wild-type (left: purple) and transgenic (right: white) petunia plants. (B) Northern blot analysis of low molecular weight RNA using CHS-A gene probe. Lane 1, 20-mer CHS-A DNA primers for a size control; lane 2, small RNA fractions from wild-type petunia; lane 3, small RNA fractions from transgenic petunia. Positions of 5S RNA and 20-mer primers are shown on the left side. siRNA signals are shown by arrows on the right side. (C) Northern blot analysis of the same blot with 5S RNA gene probe in order to show that an equal amount of small RNA fraction was loaded in lanes 2 and 3.

corresponding to RG21 (Masumura et al., 1989) was used as a template for PCR labeling using universal primers.

Protocol

Solutions

- RNA extraction buffer: 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, 1% (w/v) SDS (see Napoli et al., 1990)
- TE buffer: 10 mM Tris-HCl (pH 7.5), 1 mM EDTA
- RNA/DNA Mini Kit (QIAGEN)
- Gel-loading buffer: 2 × TBE, 40% (w/v) sucrose, 0.1% (w/v) bromophenol blue
- Denaturing PAGE gel: 15% polyacrylamide, 7 M Urea, 0.5 × TBE
- Prehybridization solution: 50% (v/v) formamide, 7.6% (w/v) SDS, 1% (w/v) N-laurylsarcosine, 0.6 μg yeast tRNA, 2% (w/v) blocking reagent (Roche)
- DIG Northern Starter Kit (Roche)
- Reaction mixture of PCR-labeling: 1 mM dATP, dGTP, dCTP, 0.65 mM dTTP, 0.35 mM Dig-11-dUTP (Roche), 1 × EX-Taq buffer, 5 U EX-Taq (TaKaRa), 20 pmol primers, template DNA
- Washing solution: 2 × SSC, 0.2% (w/v) SDS
- Equilibration buffer: 0.1 M Tris-HCl (pH 9.5), 0.1 M NaCl, 5 mM $MgCl_2$, 0.02% NaN_3

Isolating total RNA and separating low molecular weight RNA from total RNA

- Freeze plant tissues with liquid nitrogen and grind them to a powder with 500 μL of RNA extraction buffer and 150 μL of TE-saturated phenol using a mortar and pestle.[1]
- Add 250 μL of chloroform to the tube and mix the suspension by vortex.
- Centrifuge the tube at 14,000 *g* for 5 min.
- Transfer the aqueous phase to a new tube.
- Precipitate nucleic acids by adding 2.2 vol of ethanol. Incubate at -20°C for 30 min.
- Centrifuge the tube at 14,000 *g* for 15 min. Discard the supernatant.
- Dissolve the pellet in 150 μL of QRL1 solution in RNA/DNA Mini Kit (QIAGEN). Separate low molecular weight RNA from DNA and higher molecular weight RNAs according to the manufacturer's instructions.[2]

Electrophoresis and transfer

- Dissolve the precipitate of low molecular weight RNA in 15 μL of formamide. Heat the tube at 65°C for 15 min and chill on ice.
- Add 1/4 vol of gel-loading buffer.
- Separate the RNAs (20-100 μg) by denaturing PAGE at 200V for 2.5 h.
- Transfer the RNAs to Hybond-N^+ membrane (Amersham Pharmacia Biotech) by capillary blotting (Sambrook and Russell, 2001).
- Rinse the membrane with 2 × SSC for 15 min and let dry. Fix RNA on the membrane by ultraviolet cross-linking.

Notes:

1. To detect rice *glutelin* siRNAs, grind 30 developing seeds in 2 mL of 10 × TE buffer (100 mM Tris-HCl [pH 7.5], 10 mM EDTA) with a mortar and pestle. Extract nucleic acids once with 2 mL of phenol-chloroform-isoamylalcohol (25:24:1).
2. Separating low molecular weight RNA from DNA and higher molecular weight RNAs can be replaced by precipitating high molecular weight nucleic acids using polyethylene glycol (PEG) as follows:
   - Dissolve the pellet of nucleic acids in 300 μL of TE buffer.
   - Add an equal volume of PEG precipitation solution (20% PEG [MW = 8000], 2 M NaCl), mix, and keep on ice for at least 30 min.
   - Centrifuge the tube at 14,000 *g* for 15 min. Transfer the supernatant to a new tube.
   - Precipitate small RNAs by adding an equal amount of 2-propanol.

Hybridization and chemiluminescent detection

- Label the probe with digoxigenin using PCR consisting of 3 steps: denaturation at 94°C for 5 min; 30 cycles at 94°C for 30 s, 53°C for 30 s, and 72°C for 1 min; and a final extension at 72°C for 5 min (for reaction mixture, see above).
- Prehybridize the fixed membrane in prehybridization solution at 40°C for 1 h.[1]

- Denature the labeled probe by heating and chilling on ice.
- Add probe to the prehybridization solution.
- Perform the hybridization at 40°C for 12 h.
- Wash the membrane twice with washing solution at 50°C for 15 min.
- Perform the signal detection process, including blocking the membrane, reacting hybridized probes with Anti-Digoxigenin-AP Fab fragments, and washing the membrane, according to manufacturer's instructions (DIG Northern Starter Kit, Roche).
- Equibrate the membrane in equibration buffer.
- Soak the membrane in CDP-Star solution (Applied Biosystems) and expose it to x-ray film (Fuji film).

Notes:

1. Hybridization and detection were performed according to the protocol of Masuta et al. (1998) with some modifications.

Results and Discussion

We determined the presence or absence of siRNAs in transgenic petunias that showed a silenced flower color phenotype. We analyzed wild-type petunias as a negative control. Small RNA fractions extracted from these plants were separated by PAGE, blotted to nylon membranes, and hybridized with a labeled probe. Hybridization signals were detected in the silenced plants, but not in the wild-type plants (Figure 1). The hybridized RNAs migrated in the gel slightly more slowly than a 20-mer DNA oligonucleotide, indicating that the signals corresponded to siRNAs. At the hybridization stringency that allowed siRNA detection (see below), nonspecific hybridization signals appeared in the lower mobility regions, as observed in other studies (Hamilton and Baulcombe, 1999; Mette et al., 2000). The siRNA signals in Figure 1 did not appear as clear bands, probably because of the presence of polysaccharides in the small RNA fraction that affected RNA migration during electrophoresis. Alternatively, CHS-A mRNA might not be fully degraded to 21-26 nt RNA and thus appears as smeared bands. Hybridization signals of RNA more mobile than 5S RNA and less mobile than siRNAs were also detected in the silenced plants (Figure 1). These may come from intermediate products of RNA degradation produced in cells or during RNA extraction or PAGE. Regarding CHS-A transcripts in petunia, Metzlaff et al. (2000) actually detected a number of short poly(A)$^+$ RNA molecules that are thought to be the cleaved products of transcripts in silenced cells.

We also detected siRNAs in rice plants exhibiting the PTGS phenotypes of *glutelin* genes in endosperm cells (Figure 2). In this case, 2 bands, likely corresponding to short and long siRNAs (Hamilton et al., 2002), were clearly detected. Although we detected siRNAs by subjecting 20 μg of the small RNA fraction from rice endosperm and petunia flowers to PAGE, the amount of RNA required to detect siRNAs may be reduced. As a positive hybridization control, we used a mixture of 2 DNA oligonucleotides, which were used as primers for PCR labeling. We detected hybridization signals from 20 pmol of oligonucleotide DNA (10

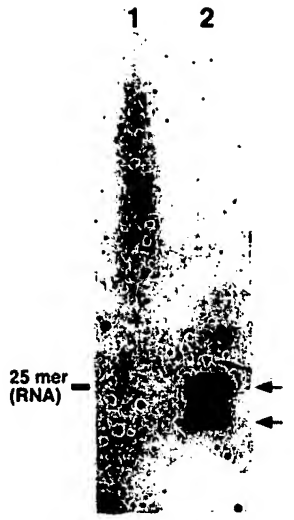

Figure 2. Northern blot analysis of low molecular weight RNA from wild-type strain and a strain that shows a silenced phenotype of rice *glutelin* genes. Lane 1, small RNA fractions from wild-type strain of rice; lane 2, small RNA fractions from silenced strain of rice. Position of a 25 nt synthetic RNA is shown on the left side. siRNA signals are shown by arrows on the right side.

pmol of each) on the membrane, suggesting that our method can detect siRNAs from small amounts of RNA.

We modified existing methods of detecting siRNAs to simplify the protocol. Hamilton and Baulcombe (1999) originally used in vitro transcribed radiolabeled RNA probes, which allowed them to distinguish sense and antisense RNAs. The presence of both sense and antisense siRNA strands has been shown in various PTGS/RNAi systems (Hamilton and Baulcombe, 1999; Hammond et al., 2000; Mette et al., 2000; Parrish et al., 2000; Yang et al., 2000; Sijen et al., 2001). siRNAs have also been detected using radiolabeled DNA probes (Ketting et al., 2001; Llave et al., 2002). We prepared probes by PCR, incorporating digoxigenin-labeled dUTPs into DNA strands. Similar results were obtained when DNA was labeled using a random priming reaction (data not shown). We also used digoxigenin-labeled RNA probes prepared by means of in vitro transcription. Stronger hybridization signals were detected using RNA probes, although the background level was often higher (data not shown). We used the labeled probes for hybridization without hydrolyzing them. Although not needed in our case, hydrolysis produces probes averaging 50 nt (Hamilton and Baulcombe, 1999) and may facilitate the detections of siRNAs. Using a radiolabeled probe, Sijen et al. (2001) obtained a result similar to ours in detecting siRNAs in petunia. We believe there are few disadvantages in using nonradiolabeled probes for siRNA detection.

In our protocol, the small RNA fraction was enriched using an ion-exchange column. This was necessary because of the limited RNA solubility and the volume of RNA solution that can be applied to electrophoresis. This process could be replaced by precipitating high molecular weight nucleic acids using 10% PEG

(MW = 8000) and 1 M NaCl and precipitating the small RNA fraction using 2-propanol. After PAGE fractionation, previous methods blotted the RNA to membranes using an apparatus such as the Bio-Rad Trans-Blot SD semidry transfer cell (Hamilton and Baulcombe, personal communication; Mette et al., 2000; Llave et al., 2002). We blotted the RNA to membranes using a conventional capillary transfer method (Sambrook and Russell, 2001). Visualizing RNA in the gel using ethidium bromide staining before and after capillary blotting indicated that the small RNA fraction was fully transferred from the gel within 5 h.

The stringency of the hybridization and posthybridization washes needed to be lower than in the standard protocol for Northern blot analysis recommended in the DIG Northern Starter Kit (Roche). We optimized the stringency by reducing the hybridization temperature and washing the membrane without changing the stringency of the hybridization or wash solutions. Hybridization was performed at 40°C, which is lower than the temperature recommended for standard Northern blot analysis (50°C). Hybridization signals were detected when the final membrane wash was done in 2 × SSC and 0.2% SDS at 50°C, whereas no signal was detected with high-stringency washes in 0.2 × SSC and 0.2% SDS at 50°C. siRNA signals were detected by exposure to x-ray film for 1 h at room temperature. Digoxigenin-labeled probes can be stored for long periods, facilitating repeated experiments. In addition, using nonradiolabeled probes avoids problems that accompany radiolabeled probes. Although the extraction of nucleic acids in the first step of this protocol is based on the method used to isolate RNA from plant tissues (Napoli et al., 1990), the remainder of the process can be applied to RNA from any source. Our method is applicable to detecting siRNAs in a variety of organisms, once nucleic acids have been properly extracted from tissues.

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siRNAs generated by recombinant human Dicer induce specific and significant but target site-independent gene silencing in human cells

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ABSTRACT

RNA interference has emerged as a powerful tool for the silencing of gene expression in animals and plants. It was reported recently that 21 nt synthetic small interfering RNAs (siRNAs) specifically suppressed the expression of endogenous genes in several lines of mammalian cells. However, the efficacy of siRNAs is dependent on the presence of a specific target site within the target mRNA and it remains very difficult to predict the best or most effective target site. In this study, we demonstrate that siRNAs that have been generated *in vitro* by recombinant human Dicer (re-hDicer) significantly suppress not only the exogenous expression of a puromycin-resistance gene but also the endogenous expression of H-*ras*, c-*jun* and c-*fos*. In our system, selection of a target site is not necessary in the design of siRNAs. However, it is important to avoid homologous sequences within a target mRNA in a given protein family. Our diced siRNA system should be a powerful tool for the inactivation of genes in mammalian cells.

INTRODUCTION

RNA interference (RNAi) is a phenomenon whereby double-stranded RNA (dsRNA) induces the sequence-dependent degradation of a cognate mRNA in animal or plant cells (1–4). The mechanism responsible for dsRNA-induced gene silencing, which proceeds via a two-step mechanism, appears to have been strongly conserved during evolution (5–8). In the first step, long dsRNAs are recognized by a nuclease in the RNase III family known as Dicer, which cleaves the dsRNA into small interfering RNAs (siRNAs) (7) of 21–23 nt. These siRNAs are incorporated into a multicomponent nuclease complex, known as RISC, that is then responsible for the destruction of cognate mRNAs (9–11).

Since dsRNAs act as inactivating agents of specific genes, they have been utilized as tools for the functional analysis of genes in a nematode, the fruit fly and plants (12–14). It has been reported that, in mammalian cells, long dsRNAs induce the sequence-specific silencing of genes in mouse embryonal carcinoma cells and embryonic stem cells (15,16). However, long dsRNAs (of >30 nt in length) activate a dsRNA-dependent protein kinase (PKR) and 2′,5′-oligoadenylate synthetase (17) in mammalian somatic cells and the activities of these enzymes lead to a non-specific reduction in levels of mRNAs.

It was reported recently that synthetic 21 nt siRNAs specifically suppressed the expression of endogenous genes in several lines of mammalian cells (18). Use of these 21 nt siRNA duplexes circumvented the activation of PKR and 2′,5′-oligoadenylate synthetase and suggested that siRNAs might be useful as gene-inactivating agents in mammals. However, the efficacy of siRNAs is dependent on identification of a specific target site within a target mRNA (19). To obtain effective siRNAs, it is necessary, although both costly and time-consuming, to design and synthesize many different siRNAs.

In this report, we demonstrate that siRNAs generated *in vitro* by recombinant human Dicer (re-hDicer) significantly suppressed not only the exogenous expression of a puromycin-resistance gene but also the endogenous expression of H-*ras*, c-*jun* and c-*fos*. As reported recently, it is possible to produce short dsRNAs using RNase III from *Escherichia coli* (20). However, since dsRNAs of 12–15 nt in length, on average, are generated by this RNase III (21), it is better to use re-hDicer, which generates a more uniform population of 21–23 nt siRNAs. Use of re-hDicer to generate siRNAs might provide a powerful tool for studies of the mechanism of RNAi and the functions of various genes in mammalian cells, with potential utility in a clinical setting.

MATERIALS AND METHODS

Cloning of the human gene for Dicer and purification of re-hDicer

Partial cDNAs for human dicer/HERNA (nucleotides 379–1657 and 1390–7037) in the pBluescript vector (Stratagene, CA) were a gift from Dr S. Matsuda of the

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University of Nagoya (22). We amplified the Dicer 5′ coding region (nucleotides 183–902) from a HeLa cDNA library by PCR with specific primers (forward primer, 5′-ATG AAA AGC CCT GCT TTG CAA CCC CT-3′; reverse primer, 5′-AGT TGC AGT TTC AGC ATT ACT CTT-3′) and then we cloned the amplified DNA into the TA cloning vector (Invitrogen, CA). Then we cloned the full-length human gene for Dicer from these partial cDNAs for human Dicer (hDicer). We digested full-length cDNA for hDicer and subcloned the blunt-ended fragment into the PinPoint™-Xa vector (Promega, Madison, WI), which contained the coding sequence for a biotin-binding region, using an *Eco*RV site. The hDicer expression plasmid was introduced into *E.coli* with 2 μM biotin and expressed upon induction with 100 μM IPTG. Then, cells were collected and pelleted by centrifugation at 5000 r.p.m. for 10 min. Cells were resuspended in lysis buffer [100 mM NaCl, 0.5 mM EDTA, 1% Triton X-100, 1 mM dithiothreitol, 2 mM phenylmethylsulfonyl fluoride, 50 mM Tris–HCl (pH 8.0)]. Then re-hDicer was purified on SoftLink™ resin (Promega) that included bound streptavidin, according to the manufacturer's protocol. For detection of re-hDicer, an aliquot of the resultant preparation of re-hDicer (0.5 μg) was fractionated by SDS–PAGE (10% polyacrylamide) and transferred to a PVDF membrane (Funakoshi Co., Tokyo, Japan) by electroblotting. Then re-hDicer was visualized with an ECL kit (Amersham Co., Little Chalfont, UK) and streptavidin-conjugated alkaline phosphatase.

Preparation of long dsRNAs and siRNAs

To generate the long dsRNA, a puromycin-resistance gene (nucleotides 1–300), the H-*ras* gene (nucleotides 370–570), the c-*jun* gene (nucleotides 1–200) and the c-*fos* gene (nucleotides 1–200) were amplified by PCR with a specific forward primer that contained a T7 promoter and a specific reverse primer that contained an SP6 promoter. Then, sense strand RNAs were generated by T7 RNA polymerase and antisense strand RNAs were generated by SP6 RNA polymerase. All siRNAs directed against puromycin-resistance mRNA and H-*ras* mRNA were synthesized by Japan Bio Service Co. Ltd (Saitama, Japan). Ten target sites (sites 1–10) were chosen for the synthetic siRNAs directed against the puromycin-resistance mRNA (site 1, 5′-ATG ACC GAG TAC AAG CCC A-3′; site 2, 5′-CTC GCC ACC CGC GAC GAC G-3′; site 3, 5′-CAC CGT CGA CCC GGA CCG C-3′; site 4, 5′-GAA CTC TTC CTC ACG CGC G-3′; site 5, 5′-GGT GTG GGT CGC GGA CGA C-3′; site 6, 5′-CAG ATG GAA GGC CTC CTG G-3′; site 7, 5′-GGA GCC CGC GTG GTT CCT G-3′; site 8, 5′-GGG TCT GGG CAG CGC CGT C-3′; site 9, 5′-CCT CCC CTT CTA CGA GCG G-3′; site 10, 5′-GCC CGG TGC CTG ACG CCC G-3′). Ten target sites (sites 1–10) were chosen for the synthetic siRNAs against H-*ras* mRNA (site 1, 5′-ATG ACG GAA TAT AAG CTT G-3′; site 2, 5′-GTT GGC GCC GGC GGT GTG G-3′; site 3, 5′-TAC GAC CCC ACT ATA GAG G-3′; site 4, 5′-AGG AGG AGT ACA GCG CCA T-3′; site 5, 5′-CAA CAC CAA GTC TTT TGA G-3′; site 6, 5′-GGA CTC GGA TGA CGT GCC C-3′; site 7, 5′-TCT CGG CAG GCT CAG GAC C-3′; site 8, 5′-GAC CCG GCA GGG AGT GGA G-3′; site 9, 5′-GCT GCGGAA GCT GAA CCC T-3′; site 10, 5′-GTG TGT GCT CTC CTG AGG A-3′). Then, to generate siRNAs, all RNAs were annealed by the standard method (6).

Processing *in vitro* by re-hDicer and purification of diced siRNAs

To examine the activity of hDicer, we mixed 10 μg of dsRNA with 1 μg of re-hDicer in 200 μl of reaction buffer [100 mM NaCl, 20 mM HEPES, 1 mM ATP, 5 mM $MgCl_2$, 50 mM Tris–HCl (pH 7.0)]. The mixture was incubated for 30 min at 37°C. Then, 20 μl of the reaction mixture were fractionated by electrophoresis on a non-denaturing 12% polyacrylamide gel. Bands of RNA were detected with Syber™ green II reagent (Nippon Gene, Toyama, Japan). We recovered siRNAs of 21–23 nt in length from the reaction mixture using a QIAquick™ nucleotide-removal kit (Qiagen, Hilden, Germany). The siRNAs were precipitated in ethanol and then dissolved in TE buffer. The concentration of diced siRNAs was determined by monitoring absorbance at 260 nm.

Transfection of cells and assay of cell viability

HeLa cells were cultured in DMEM supplemented with 10% fetal bovine serum. Transfections with either 20 nM siRNAs or diced siRNAs were performed using the Oligofectamine™ reagent (Invitrogen) in accordance with the manufacturer's instructions. Cell viability was determined with trypan blue as described.

Western blotting

HeLa cells that had been transfected with individual siRNAs were harvested. Proteins were resolved by SDS–PAGE (10% polyacrylamide) and transferred to a PVDF membrane (Funakoshi Co.) by electroblotting. Immune complexes were visualized with an ECL™ kit, using specific polyclonal antibodies against H-Ras (Oncogene Research Products, San Diego, CA), N-Ras (Oncogene Research Products), K-Ras (Oncogene Research Procducts), c-Jun (Santa Cruz Biotechnology, Santa Cruz, CA), c-Fos (Santa Cruz Biotechnology) and actin (Oncogene Research Products), as an endogenous control. Quantitation was performed by densitometry and NIH Image Analysis.

RESULTS

Cloning of a human gene for Dicer and purification and characterization of re-hDicer

To prepare heterogeneous siRNAs that could target various sites in a specific target mRNA, we used hDicer, which participates in RNAi in human cells. Initially, to generate re-hDicer using a bacterial expression system, we cloned the full-length human gene for Dicer using partial cDNAs for hDicer (see Materials and Methods; 7,22). The full-length cDNA was then subcloned into the PinPoint™-Xa vector that contained the coding region for the biotin-binding region of a biotin ligase for subsequence purification of the recombinant protein.

We introduced the hDicer expression plasmid (phDicer) into *E.coli* and, with biotin in the culture medium, re-hDicer was expressed upon induction with IPTG. The re-hDicer with bound biotin was purified by a 'pull-down' method with beads to which streptavidin had been bound (see Materials and Methods) and analyzed by SDS–PAGE, after which re-hDicer was detected by western blotting with visualization using alkaline phosphatase-conjugated streptavidin. As shown in

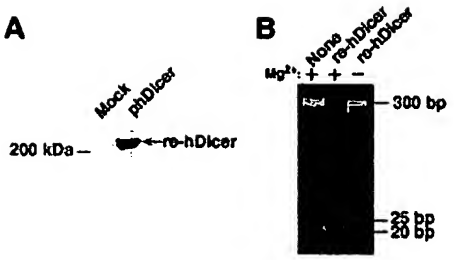

Figure 1. (A) Detection of the hDicer-mediated generation of diced siRNAs. Re-hDicer was synthesized in *E.coli* and detected by western blotting analysis, as described in the text. The preparation of re-hDicer (0.5 μg) was fractionated by SDS–PAGE (10% polyacrylamide) and transferred to a PVDF membrane by electroblotting. Then re-hDicer was visualized with an ECL kit and streptavidin-conjugated alkaline phosphatase. Mock, preparation from cells transfected with the empty plasmid; phDicer, preparation from cells infected with an expression plasmid that encoded hDicer. (B) Generation of siRNAs by re-hDicer. Long dsRNA (10 μg) was mixed with 1 μg of re-hDicer in 200 μl of reaction buffer, with or without 5 mM $MgCl_2$. The reaction mixtures were incubated for 30 min at 37°C. Then 20 μl of each reaction mixture were fractionated by electrophoresis in a 12% non-denaturing polyacrylamide gel. siRNAs of 20–25 nt in length were detected with Syber™ green II.

Figure 1A, we detected re-hDicer with a biotin tag by western blotting. The putative molecular mass of the biotin-labeled re-hDicer was ~220 kDa. Mock transfection with a vector that did not include the cDNA for hDicer did not yield the corresponding protein, confirming the production of re-hDicer in this expression system in *E.coli*.

Next, to determine whether the re-hDicer had RNase III activity, we performed a processing assay *in vitro* using long dsRNAs that had been generated by transcription *in vitro*. To construct long dsRNAs for use as substrates, we amplified a puromycin-resistance gene (nucleotides 1–300) by PCR using a specific forward primer that contained a T7 promoter and a specific reverse primer that contained an SP6 promoter. Then sense strand RNAs were generated by T7 RNA polymerase and antisense strand RNAs were generated by an SP6 RNA polymerase. These transcribed RNAs were annealed by the standard method (6). Then, the long dsRNAs and re-hDicer were combined and incubated in an appropriate reaction mixture (see Materials and Methods) for 30 min at 37°C. As shown in Figure 1B, siRNAs of 20–25 nt in length were generated in the presence of re-hDicer. In contrast, in the absence of either re-hDicer or Mg^{2+} ions, no such siRNAs were detected (lanes 1 and 3). Thus, re-hDicer exhibited Mg^{2+}-dependent dsRNA processing activity. It was reported recently that recombinant hDicer in insect cells also exhibits RNase III activity (23,24).

Effects of diced siRNAs on expression of an exogenous puromycin-resistance gene

In mammalian cells, synthetic 21 nt siRNAs suppress the expression of both reporter genes and endogenous genes to a significant extent (18,19,25). While long dsRNAs (>30 nt) activate PKR and 2′,5′-oligoadenylate synthetase, use of these shorter siRNAs circumvents the activation of these enzymes and activates the RNAi pathway. Thus, these siRNAs might be expected to be useful for the sequence-specific silencing of gene expression. However, the dependence of the activity of siRNAs on the target site has been reported (19), and it remains very difficult to predict the best and most effective target site. Since the dependence of the activity of siRNAs on the target site was examined only in the case of the human gene for tissue factor (19), we further investigated the dependence on target site using siRNAs targeted to a puromycin-resistance gene and directed against 10 target sites within this gene (Fig. 2A and B). We used siRNA targeted to GL3-luciferase as a control.

To prepare diced siRNAs, we transcribed both sense and antisense strands of a partial mRNA for puromycin resistance (nucleotides 1–300) *in vitro*, using T7 and SP6 RNA polymerases (Fig. 1B). After the dicing of dsRNAs *in vitro*, we purified the diced siRNAs using a QIAquick™ nucleotide-removal kit. Next, we introduced each siRNA (20 nM) and the expression plasmid for a puromycin-resistance gene into HeLa cells using the Oligofectamine™ reagent. After 36 h, we treated the HeLa cells with puromycin and determined cell viability using trypan blue. The efficiency of transfection of each siRNA was measured with the luciferase gene from *Renilla* as a reporter gene.

As shown in Figure 2C, in the presence of siRNA targeted to GL3-luciferase, cell viability remained the same as that of wild-type HeLa cells. The viability of cells that had been treated with site 2-, site 3-, site 4-, site 5- and site 10-specific siRNAs was significantly lower than that of wild-type cells that harbored the puromycin-resistance gene. In contrast, site 1-, site 6-, site 7-, site 8- and site 9-specific siRNAs had lower growth-inhibitory activity. These results indicated that the efficiency of siRNAs that were specific for the puromycin-resistance gene depended on the target site within the target mRNA.

As we had anticipated, diced siRNAs that corresponded to unlimited target sites within the specific mRNA were more effective than any specific siRNA or a mixture of site 1-, site 2-, site 3-, site 4- and site 5-specific siRNAs. When we analyzed the GC content of target sites and the secondary structure of the target gene, as predicted with the mfold program (Fig. 2B) (26), we failed to recognize a clear correlation between either secondary structure or GC content (Table 1) and effective and less effective target sites. In the case of the target gene, siRNAs targeted to regions from position 20 to position 280 and near the 3′ end of the target gene were more effective than others that we tested.

Effects of diced siRNAs on expression of an endogenous H-*ras* gene

To examine the effects of diced siRNAs on expression of an endogenous gene, we selected the H-*ras* gene as a target. The H-*ras* gene is a member of the *ras* family, which also includes K-*ras* and N-*ras* (27). Again, to check the dependence on target site of the effects of siRNAs, we selected 10 target sites in the H-*ras* gene (Fig. 3A and B). For construction of diced siRNAs, we searched for a region of low homology to other members of the *ras* family in the H-*ras* gene. We selected such a region of the H-*ras* gene (nucleotides 370–570) as the long dsRNA substrate. Diced siRNAs targeted to the H-*ras* gene were generated by the same method as described above. Each individual siRNA (20 nM) was introduced into HeLa cells using the Oligofectamine™ reagent. After 72 h, cells were

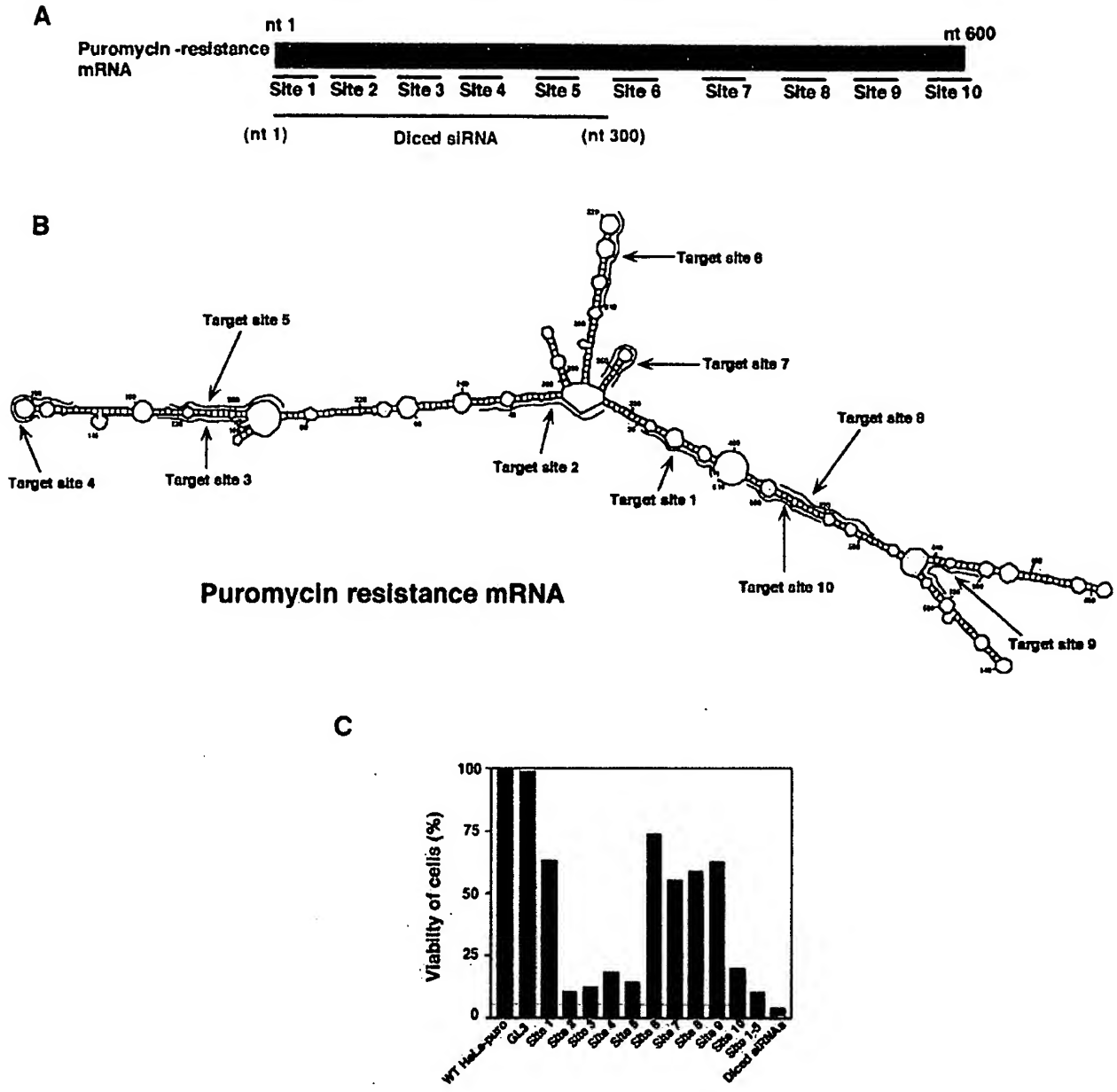

Figure 2. Suppression of the expression of an exogenous puromycin-resistance gene. (**A**) Ten sites (sites 1–10) were chosen as targets for synthetic siRNAs, as detailed in the text. For preparation of diced siRNAs, long dsRNAs corresponding to the 5′ region of the puromycin-resistance gene (nucleotides 1–300) were generated and treated with re-hDicer. (**B**) The secondary structure of the puromycin-resistance mRNA as predicted by the mfold program (24). (**C**) Diced siRNAs were the most effective suppressors of the expression of the exogenous puromycin-resistance gene (as indicated by the bar on the far right of the histogram). The efficiency of transfections with siRNAs was monitored with a reporter gene for luciferase from *Renilla*.

collected and total proteins were extracted. Levels of H-Ras protein were monitored by western blotting with specific antibodies. We determined levels of actin similarly as an endogenous control. Quantitation of proteins was performed by densitometry and NIH Image Analysis.

As shown in Figure 3C, the levels of H-Ras in cells that had been treated with site 2-, site 3-, site 4-, site 5- and site 9-specific siRNAs were significantly lower than that in wild-type HeLa cells. In contrast, site 1-, site 6-, site 7- and site 8-specific siRNAs had lower inhibitory activity. We normalized the results by reference to levels of actin and the results indicated that the efficiency of the siRNAs depended on the target site in the endogenous H-*ras* gene. In the case of both the puromycin-resistance gene and the H-*ras* gene, synthetic siRNAs targeted to the central regions of the genes were less effective. As for the diced siRNAs, they were

Table 1. GC contents (%) of the target site of each siRNA and levels of inhibition of gene expression

Target site	Puromycin-resistance mRNA		H-*ras* mRNA	
	GC (%)	Inhibition (%)	GC (%)	Inhibition (%)
1	52.6	37.2	36.0	63.2
2	78.9	88.6	78.9	81.7
3	78.9	86.9	52.6	78.6
4	63.1	79.5	57.8	87.8
5	73.6	84.6	42.0	70.5
6	73.6	24.8	68.4	45.3
7	73.6	45.1	68.4	23.6
8	78.9	40.5	73.6	36.8
9	68.4	36.8	63.1	74.2
10	84.0	78.2	57.8	82.1

significantly more effective than all the other siRNAs tested and than a mixture of siRNAs (site 7-, site 8-, site 9- and site 10-specific siRNAs) targeted to the H-*ras* gene. The levels of K-Ras and N-Ras proteins in cells that had been treated with diced siRNAs were, as anticipated, similar to those in wild-type HeLa cells (Fig. 3D). Thus, the diced siRNAs did not affect the expression of either the K-*ras* or the N-*ras* gene.

A structural analysis (Fig. 3B) similar to that shown in Figure 2B again failed to indicate any correlation between GC content (Table 1) and the extent of inhibition by siRNAs. Since results obtained in a previous study suggested that the effects of siRNAs, oligonucleotides and ribozymes might share some common features (28), it seems likely that more experimental data related to the accessibility of target mRNAs *in vitro* and *in vivo* (rather than computer-predicted secondary structures, as shown, for example, in Fig. 3B) are needed for a more precise examination of such putative relationships. In the case of H-*ras*, again, siRNAs targeted to certain regions (from position 20 to 280 and near the 3′ end) in the target gene were more effective than others, and diced siRNAs were again much more effective than individual siRNAs or a mixture of such siRNAs (Fig. 3C). These results resembled those obtained with the puromycin-resistance gene. It is possible that the higher activity of the diced siRNAs than that of individual siRNAs might simply have been due to an additive effect of targeting multiple sites along a message. However, the results obtained with the mixture of siRNAs (site 7-, site 8-, site 9- and site 10-specific siRNAs) failed to support this interpretation. It is more likely that the effects of siRNAs are changed by even a small change in the target site (of even just a few nucleotides) and, thus, diced siRNAs appeared to include some populations that were significantly more effective than the combined individual siRNAs. The observations suggest the clear advantages of diced siRNAs over individual synthetic siRNAs, at least in the experiments carried out in this study.

Diced siRNAs directed against c-*jun* and c-*fos* mRNAs

Finally, to confirm the effects of diced siRNA, we generated diced siRNAs against c-*jun* mRNA and c-*fos* mRNA, respectively. We introduced these diced siRNAs into HeLa cells and, 72 h later, we collected the cells and extracted the total proteins. The levels of the c-Jun and c-Fos proteins were examined by western blotting with specific antibodies. As shown in Figure 4, the level of c-Jun in cells that had been transfected with diced siRNAs was significantly lower than that in wild-type HeLa cells. The levels of actin were similar in both transfected and wild-type cells. We obtained analogous results using diced siRNAs directed against c-*fos* mRNA. Our results confirmed the high potential utility of diced siRNAs in the silencing of specific genes in human cells.

DISCUSSION

In this study, we developed an effective gene-silencing method, which we refer to as diced siRNA technology, using re-hDicer. RNAi has been shown to be a powerful tool for studies of gene function in a nematode, the fruit fly and plants (12–14). Effective sequence-specific gene silencing in several animals requires dsRNA of >150 bp (8,9,29,30). However, in mammals, long dsRNAs (>30 nt) induce the non-specific reduction of gene expression via the activation of PKR. Synthetic siRNAs that can effectively suppress the expression of endogenous genes have been reported by several groups (18,19,25). However, the efficiency of siRNAs depends on a specific target site within the target gene (Figs 2 and 3).

In RNAi, siRNAs become associated with RISC and function as guide RNAs in the search for target sites (3). However, we do not yet know how the siRNA–RISC complex can gain access to its target site and how cleavage at the target site occurs. In the nematode *Caenorhabditis elegans*, a RNA-directed RNA polymerase (RdRP) chain reaction with siRNA amplifies the interference that is caused by a small amount of 'trigger' dsRNA (31). Thus, siRNA acts not only as a guide but also as a primer and long dsRNAs are generated by RdRP. These long dsRNAs are then cleaved into fragments of ~21 nt in length by Dicer.

Since accessibility of the siRNA might depend on the secondary structure of the target mRNA, we have to design and synthesize many different siRNAs to obtain effective siRNAs, and, at present, success depends on trial and error. To overcome these problems, we utilized re-hDicer for dicing of long dsRNAs *in vitro* and no longer needed to select a target site. However, it remained important to avoid the selection of sequences homologous to those of other members of the family to which the target protein belonged. Using our method, we succeeded in suppressing the expression of several

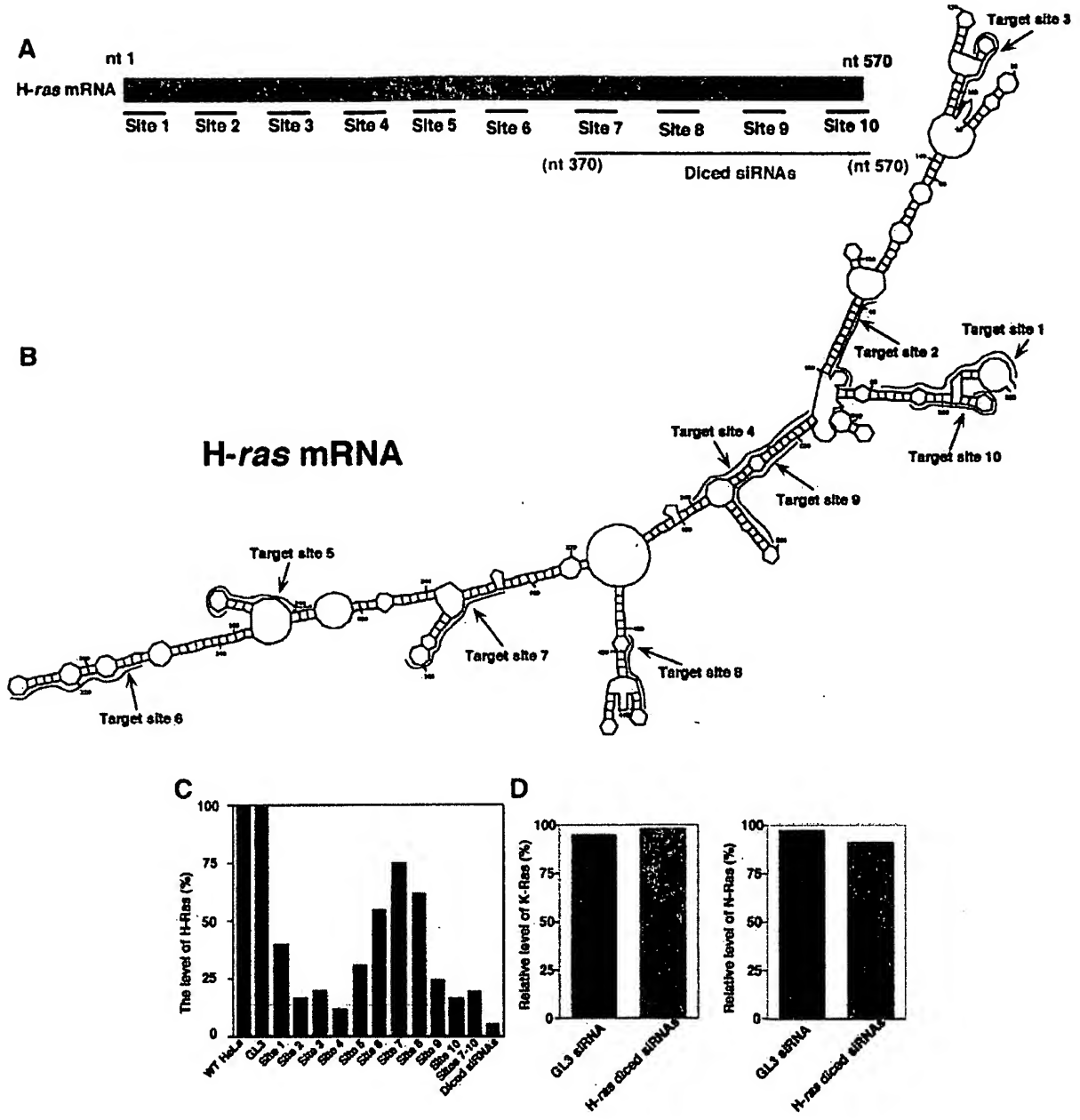

Figure 3. Suppression of the expression of the endogenous H-*ras* gene. (**A**) Ten sites (sites 1–10) were chosen as targets for synthetic siRNAs, as detailed in the text. For the preparation of diced siRNAs, long dsRNAs corresponding to the 3′ region of the H-*ras* gene (nucleotides 370–570), which exhibited weak homology to the sequences of related *ras* genes, were generated and treated with re-hDicer. (**B**) The secondary structure of H-*ras* mRNA, as predicted by the mfold program (24). (**C**) Diced siRNAs were the most effective suppressors of the expression of the endogenous H-*ras* gene (as indicated by a bar on the far right of the histogram). The results were normalized by reference to levels of actin, as a control. (**D**) Effects of diced siRNAs targeted to the H-*ras* gene on the expression of the related K-*ras* and N-*ras* genes. K-Ras and N-Ras were detected by western blotting analysis with specific antibodies. Levels were quantitated by densitometry and NIH Image Analysis.

endogenous genes that included H-*ras*, c-*jun* and c-*fos*, as shown in Figures 3 and 4. It appears, therefore, that the diced siRNA technology might be a powerful tool for the functional analysis of genes of interest, as well as for inactivation of specific genes in a clinical setting.

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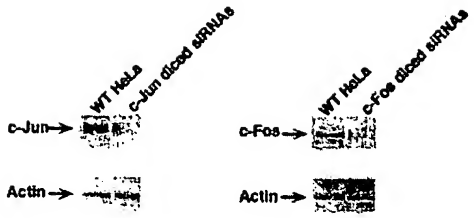

Figure 4. Suppression of the expression of the endogenous c-*jun* and c-*fos* genes by diced siRNAs. For the preparation of diced siRNAs, long dsRNAs corresponding to the 5′ region of the c-*jun* gene (nucleotides 1–200) and the c-*fos* gene (nucleotides 1–200) were generated and treated with re-hDicer. Both c-Jun and c-Fos were detected by western blotting analysis with specific antibodies. Actin was used as an endogenous control. WT-HeLa, wild-type HeLa cells; c-Jun (c-Fos) diced siRNs, siRNAs generated from c-*jun*-specific (c-*fos*-specific) dsRNA.

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